Natural products with antifungal properties as alternative to copper in agriculture

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Justine Fabienne Ramseyer

aus Eggiwil, Bern

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auf Antrag von

Prof. Dr. Matthias Hamburger

Dr. Emerson Ferreira Queiroz

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Prof. Dr. Martin Spiess

Dekan

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To my dear Julien,

To my family and friends

"All the pests that out of earth arise,
the earth itself antidote supplies."

Lithica poem (ca. 400 B. C.)

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LIST OF ABBREVIATIONS

AAAS	American Association for the Advancement of Science
ACD	Advanced Chemistry Development
ANOVA	Analysis of Variance
ASE	Accelerated Solvent Extraction
ATP	Adenosine Triphosphate
B3LYP	Becke Three-Parameter Exchange, Lee-Yang-Parr Correlation
BC	Benzyl cinnamate
BP	Balsam of Peru
Bt	*Bacillus thurigiensis*
CB	Coniferyl Benzoate
CC	*p*-Coumaryl Cinnamate
CIFLORPAN	Center for Pharmacognostic Research on Panamian Flora
COSY	Correlation Spectroscopy
CPCM	Conductor-like Polarisable Continuum Model
DEPT	Distortionless Enhancement by Polarisation Transfer
DFT	Density Function Theory
DHEF	Dehydroeffusol
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOI	Digital Object Identifier
DT	Dissipation Time
EC	Emulsifiable Concentrate
EC_{50}	Half Maximal Effective Concentration
ECD *spectroscopy*	Electronic Circular Dichroism *spectroscopy*
ED	Effective Dose
EPPO	European and Mediterranean Plant Protection Organization
ESI	Electrospray Ionisation
EtOH	Ethanol
FiBL	Forschungsinstitut für biologischen Landbau (Research Institute of Organic Farming)
GABA	γ-Aminobutyric Acid
GMO	Genetically Modified Organism
HMBC	Heteronuclear Single-Quantum Correlation
HPLC	High Performance Liquid Chromatography
HPPD	*p*-Hydroxyphenylpyruvate Dioxygenase

HRESIMS	High Resolution Electrospray Ionisation Mass Spectrometry
HR-MS	High Resolution Mass Spectrometry
HSQC	Heteronuclear Single-Quantum Correlation
IFOAM	International Federation of Organic Agriculture Movements
IM	*Iryanthera megistocarpa*
IPM	Integrated Pest Management
JE	*Juncus effusus*
LD_{50}	Median Lethal Dose
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MO	*Magnolia officinalis*
MS	Mass Spectrometry
NAChR	Nicotinic Acetylcholine Receptor
NADH	Nicotinamide Adenine Dinucleotide
NMR *spectroscopy*	Nuclear Magnetic Resonance *spectroscopy*
NOESY	Nuclear Overhauser Effect Spectroscopy
NP	Normal Phase
OPLS	Optimized Potential for Liquid Simulations
PDA	Photodiode Array
PE	Petroleum Ether
Pv	*Plasmopara viticola*
RH	Relative Humidity
ROESY	Rotating Frame Nuclear Overhauser Effect Spectroscopy
RP	Reverse Phase
SAR	Systemic Acquired Resistance
SB	Siam benzoin
SCRF	Self-Consistent Reaction Field
SD	Standard Deviation
SumB	Sumatra benzoin
TD-DFT	Time Dependant Density Functional Theory
TLC	Thin Layer Chomatography
TRPV *channels*	Vanilloid-type Transient Receptor Potential c*hannels*
TXI	Triple Resonance Probe
UV	Ultraviolet
Vi	*Venturia inaequalis*
VL	*Verbesina lanata*
WP	Wettable Powder

SUMMARY

Copper salts are widely used in agriculture due to their activity against a broad range of plant pathogens. However, copper accumulates in soils, leading to ecological issues. Therefore, there is a strong demand for substitutes, especially in organic farming, where no efficient alternatives to copper are currently available.

An in-house library containing more than 3000 extracts from fungal and plant origin was screened *in vitro* against three major agricultural pathogens, specifically grapevine downy mildew (*Plasmopara viticola*), potato and tomato late blight (*Phytophthora infestans*), and apple scab (*Venturia inaequalis*). From this screening, several plant extracts with significant activity were selected for further investigation in this thesis. They include the ethyl acetate extracts of *Juncus effusus* (Juncaceae) medulla, *Magnolia officinalis* (Magnoliaceae) bark, *Verbesina lanata* (Asteraceae) leaves, *Iryanthera megistocarpa* (Myristicaceae) leaves, and the petroleum ether extracts of *Styrax tonkinensis* and *Styrax paralleloneurum*/*benzoin* (Styracaceae) resins. The active compounds were identified by an approach referred to as HPLC-activity profiling. Subsequently, the identified compounds were isolated by several chromatographic methods and characterised by diverse spectroscopic techniques, such as HR-ESI-MS, 1D and 2D microprobe NMR, ECD, optical rotation, and X-ray crystallography.

The ethyl acetate extract of *J. effusus* medulla showed strong *in vitro* inhibitory activity against the three aforementioned pathogens. The active compound was identified as dehydroeffusol. On grapevine and apple seedlings, efficacies up to 100 % were reached with the extract and the purified compound.

S. tonkinensis resin (Siam benzoin) and Sumatra benzoin (resin from *Styrax paralleloneurum* or *Styrax benzoin*) exhibited promising *in vitro* inhibitory activity against the three pathogens. On grapevine seedlings, Siam benzoin and Sumatra benzoin dissolved in ethanol were reaching an efficacy of 100% at a concentration of 1 mg/mL. The active compounds were coniferyl benzoate in Siam benzoin and *p*-coumaryl cinnamate in Sumatra benzoin. The activities of the two resins were also significant against *V. inaequalis* and *Marssonina coronaria* on apple seedlings, and against *P. infestans* on tomato seedlings. In field trials on grapevine, Siam benzoin reduced the infestation by downy mildew (*P. viticola*) and powdery mildew (*Uncinula necator*, anamorph *Oidium tuckeri*). These results led to the

submission of an European Patent Application in view of a potential commercial development.

The ethyl acetate extract of *M. officinalis* bark showed high *in vitro* activity against the three pathogens. The active compounds were identified as the two neolignans, magnolol and honokiol. The efficacies on grapevine and apple seedlings were similar to copper, whereas on tomato seedlings the efficacy was lower. The activity against apple scab could not be confirmed on field trials. In contrast, the efficacy of *M. officinalis* extract formulated as a wettable powder was equivalent to the reference organic fungicide.

The ethyl acetate extract of *V. lanata* leaves exhibited promising *in vitro* activity against *P. viticola*. At a concentration of 1 mg/mL, the extract reduced the leaf surface infestation by 82% compared to the non-treated control. Sixteen eudesmane sesquiterpenes with a cinnamoyloxy group were isolated. Among them, eight were new congeners. Nine of these compounds were tested *in vitro* against *P. viticola* and five showed MIC_{100} values <10 μg/mL.

The ethyl acetate extract of *I. megistocarpa* leaves exhibited good activity *in vitro* against the three pathogens. The leaf surface infestation on seedlings at an extract concentration of 1 mg/mL was reduced by 87% on grapevine and by 68% on tomato. Tests on apple seedlings are ongoing. From the extract, two dihydrochalcones and eight flavonolignans including several stereoisomers were isolated. Against each pathogen, the two most active compounds showed MIC_{100} values $\leq$2.3 μg/mL. Seven of the flavonolignans possess three different planar structures with several configurations along a side chain. Due to the possible free rotation, their stereochemistry could not be established by NMR analysis. Suitable crystals were only obtained for one compound allowing the determination of its relative configuration by X-ray diffraction analysis. Further crystallisation attempts should be performed with the other compounds, possibly after the preparation of suitable derivatives.

The results of this thesis confirm that plant-derived products represent promising candidates for the development of new organic pesticides which could enable to significantly reduce the use of copper. In further steps, toxicity studies will have to be performed with the plant extracts and pure compounds to ensure product safety. Further field trials with optimised extract formulations should be also carried out.

ZUSAMMENFASSUNG

Kupfersalze werden wegen ihres breiten Aktivitätsspektrums gegen pflanzliche Pathogene in vielen Bereichen der Landwirtschaft eingesetzt. Kupfer reichert sich im Boden an, was zu ökologischen Problemen führt. Daher herrscht ein starker Bedarf nach Ersatzprodukten, besonders in der biologischen Landwirtschaft, wo effiziente Alternativen zum Kupfer bisher fehlen.

Eine interne Bibliothek aus über 3000 Extrakten von Pilzen und Pflanzen wurde *in vitro* gegen drei landwirtschaftliche Hauptpathogene getestet, nämlich gegen den Falschen Mehltau der Weinrebe (*Plasmopara viticola*), die Krautfäule der Tomate und der Kartoffel (*Phytophthora infestans*), sowie den Apfelschorf (*Venturia inaequalis*). Aus diesem Screening wurden Extrakte, welche eine signifikante Aktivität aufwiesen, für weitere Untersuchungen im Rahmen dieser Arbeit ausgewählt. Dabei handelt es sich um Ethylacetat-Extrakte aus *Juncus effusus* (Juncaceae) Mark, *Magnolia officinalis* (Magnoliaceae) Rinde, *Verbesina lanata* (Asteraceae) Blättern, *Iryanthera megistocarpa* (Myristicaceae) Blättern und Petrolether-Extrakte aus *Styrax tonkinensis* und *Styrax paralleloneurum*/*benzoin* (Styracaceae) Harzen. Die aktiven Verbindungen wurden mittels dem sogenannten HPLC-activity profiling identifiziert. Anschliessend wurden sie mittels verschiedener chromatographischer Methoden isoliert und mithilfe spektroskopischer Techniken charakterisiert. Zur Anwendung kamen HR-ESI-MS, 1D und 2D microprobe NMR, ECD, Optische Rotation und Röntgen Kristallographie.

Der Ethylacetat-Extrakt aus *J. effusus* Mark zeigte *in vitro* eine stark inhibierende Aktivität gegen die drei zuvor genannten Pathogene. Die aktive Verbindung wurde als Dehydroeffusol identifiziert. Der Extrakt wie auch die isolierte Verbindung zeigten einen bis zu 100%igen Wirkungsgrad bei Weinreben- und Apfelsämlingen.

S. tonkinensis Harz (Siam benzoin) und Sumatra benzoin (Harz der *Styrax paralleloneurum* oder *Styrax benzoin*) zeigten eine vielversprechende inhibitorische Aktivität gegen die Pathogene *in vitro*. In Ethanol gelöstes Siam benzoin und Sumatra benzoin erreichten einen Wirkungsgrad von 100% bei einer Konzentration von 1 mg/mL auf den Weinrebensämlingen. Bei den aktiven Verbindungen handelt es sich um Coniferyl Benzoat in Siam benzoin und *p*-Coumaryl Cinnamat in Sumatra benzoin. Die Aktivitäten der zwei Harze sind ebenfalls signifikant gegen *V. inaequalis*, *Marssonia coronaria* auf Apfelsämlingen und

gegen *P. infestans* auf Tomatensämlingen. In Feldversuchen reduzierte Siam benzoin den Befall durch Falschen (*P. viticola*) und Echten Mehltau (*Uncinula necator*, anamorph *Oidium tuckeri*) an den Weinreben. Diese Ergebnisse führten zu einer Patentanmeldung beim Europäischen Patentamt in Hinblick auf eine mögliche kommerzielle Entwicklung.

Der Ethylacetat-Extrakt aus *M. officinalis* Rinde zeigte ebenfalls eine hohe Aktivität gegen die drei Pathogene *in vitro*. Die aktiven Verbindungen wurden als die Neolignane Magnolol und Honokiol identifiziert. Der Wirkungsgrad auf Weinreben- und Apfelsämlingen war gleich dem Wirkungsgrades des Kupfers und auf Tomatensämlingen etwas geringer. Die Aktivität gegen Apfelschorf konnte in den Feldversuchen nicht bestätigt werden. Im Gegensatz dazu war der Wirkungsgrad des *M. officinalis* Extraktes (in Form eines benetzbaren Pulvers) equivalent zur biologischen Fungizid Referenz.

Der Ethylacetat-Extrakt aus *V. lanata* Blättern zeigte eine vielversprechende Aktivität gegen *P. viticola in vitro*. Bei einer Konzentration von 1 mg/mL wurde eine Reduktion um 82% des Befalls der Blätteroberflächen beobachtet im Vergleich zur unbehandelten Kontrollgruppe. Sechzehn Eudesmane Sesquiterpene mit einer Cinnamoloxy-Gruppe einschliesslich acht neue Derivate wurden isoliert. Neun der isolierten Verbindungen wurden *in vitro* gegen *P. viticola* getestet und zeigten MIC_{100}Werte <10 μg/mL.

Der Ethylacetat-Extrakt aus *I. megistocarpa* Blättern zeigte *in vitro* eine gute Aktivität gegen die drei Pathogene. Der Befall der Blattoberflächen der Sämlinge wurde bei der Weinrebe um 87% und bei der Tomate um 67% reduziert bei einer Extraktkonzentration von 1 mg/mL. Untersuchungen an den Apfelsämlingen sind im Gange. Aus dem Extrakt wurden zwei Dihydrochalcone und acht Flavonolignane, inklusive mehrerer Stereoisomere, isoliert. Die zwei aktivsten Verbindungen erreichten MIC_{100} Werte $\leq$2.3 μg/mL bei allen drei Pathogenen. Unter den Flavonolignanen gab es sieben Substanzen, welche drei verschiedene planare Strukturen mit mehreren Konfigurationen an der Seitenkette aufwiesen. Die NMR-Analyse liess aufgrund möglicher freier Rotationen entlang der Seitenkette keinen Schluss auf die relative Konfiguration zu. Adäquate Kristalle konnten nur aus einer Verbindung gewonnen werden, um eine Bestimmung der relativen Konfiguration mittels Röntgenbeugungsanalyse durchzuführen. Weitere Versuche, verwertbare Kristallstrukturen zu erhalten, sollten unternommen werden, gegebenenfalls nach Aufbereitung geeigneter Derivate.

Die Ergebnisse dieser Arbeit bestätigen, dass Produkte pflanzlichen Ursprungs vielversprechende Kandidaten für die Entwicklung von neuen biologischen Pestiziden liefern. Dies könnte erheblich zur Reduktion des Gebrauchs von Kupfer beitragen. Um die Produktsicherheit der pflanzlichen Extrakte und reinen Verbindungen sicherzustellen, sollten in Zukunft Toxizitätsstudien durchgeführt werden. Ebenso empfehlen sich weitere Feldstudien mit optimierten Formulierungen der Extrakte.

1. AIM OF THE WORK

In agriculture, the production yields were dramatically increased during the last 50 years mainly due to the use of irrigation, high-yielding crop varieties, fertilisation, and pesticides. Nevertheless, this intensification has not been without side effects on the environment [1]. Especially the widespread use of chemical pesticides has led to acute and chronic pollutions with several deleterious impacts [2].

In organic farming, copper as pesticide is still permitted. This is mainly due to the absence of efficacious alternatives [3]. Despite the reduced doses which are applied compared to traditional agriculture [4, 5], copper accumulates in soils, leading to the imbalance of their ecology [6].

In this context, the purpose of this thesis was to find natural products with antifungal activity that could be further developed as substitute to copper. The project was initiated through a collaboration with the Research Institute of Organic Agriculture (FiBL, Forschungsinstitut für biologischen Landbau) based in Frick, Switzerland.

An in-house library containing over 3000 extracts from approximately 100 fungi and 800 plants was screened *in vitro* against three major agricultural pathogens, namely *Plasmopara viticola* (grapevine downy mildew), *Phytophthora infestans* (tomato and potato late blight), and *Venturia inaequalis* (apple scab). Several highly active plants in the screening have been selected and further investigated: *Juncus effusus* (Juncaceae), *Styrax tonkinensis* and *Styrax parralleloneurum*/*benzoin* (Styracaceae), *Magnolia officinalis* (Magnoliaceae), *Verbesina lanata* (Asteraceae), and *Iryanthera megistocarpa* (Myristicaceae). For plant selection, further criteria, such as novelty of the antifungal activity in the taxon, potential availability of the plant material on a large scale, toxicity reports, if available, and previous chemical investigations have been also taken into consideration.

In a first step activity will be tracked in the extracts by HPLC-based activity profiling, a procedure which combines biological activity data with chemoanalytical information. The active compounds will be then isolated by a combination of chromatography techniques and their structures elucidated by spectroscopic methods including UV, MS, NMR, ECD, optical rotation, and X-ray crystallography [7]. The MIC_{100} values of purified compounds and crude extracts will be then determined *in vitro* on the aforementioned three pathogens.

Once the active constituents have been identified, the activity of the extract and selected pure compounds will be assessed on grapevine, apple, and/or tomato seedlings under semi-

controlled conditions. Finally, the most promising plant extracts will be tested under field conditions. For this, large amounts of extract should be produced and appropriate formulations be developped.

In this thesis, after an introduction on the different topics, the results obtained on these several plants are being discussed in details.

2. INTRODUCTION

2.1. Natural products as a source of pesticides

The world population is going to reach 9.8 billion in 2050 [8]. Therefore, food security and sustainability are major concerns for every government [1, 9-12]. A huge productivity improvement is needed, since food supply would have to be increased by 70%, essentially on currently cropped areas [13].

Serious agricultural losses are triggered by diseases, insects, and weeds. Jointly, they are estimated to impact or destroy the crop production worldwide by 31 to 42% annually [14]. The development of pesticides has improved production yields [1, 12, 15, 16]. However, pesticides are likely to be harmful to non-target organisms, animals, humans, and the environment, since they should be biologically active or toxic to be effective against the target pests. Consequently, appropriate protection measures should be taken to avoid the exposure, the dose should be calculated carefully, and the less toxic effective product should be utilised [17-20].

Nevertheless, the extensive use of chemicals in agriculture (about 3 billion kg yearly worldwide [21]) has led to acute or chronic pollution with negative effects on the whole ecosystem and humans [1, 2, 12-15, 20, 22-31]. In a review written by Pimentel [21], the different impacts of pesticides are discussed. First of all, acute poisonings are estimated every year to more than 26 million cases. Among them, approximately 3 million result in hospitalisation, about 750’000 lead to chronic illnesses, and around 220’000 are fatal. The chronic effects are of neurological, reproductive, and respiratory nature. Moreover, repeated exposure can lead to cancer. Another issue is the residues in food. The majority of supermarket products have detectable amounts of pesticides. Besides these, the ground and surface waters also get contaminated. Moreover, the chemicals impact biodiversity, natural pest enemies (parasites and predators), fish, birds, mammals, microbes, invertebrates, and essential pollinators. Another issue is the development of resistance. Extensive information about the costs of pesticides is available in a vast review published by Bourguet and Guillemaud in 2016 [32] and in a specific work focussed on the United Kingdom published by Pretty *et al.* in 2000 [33].

Consequently, search for safer substitutes with different modes of action is of high importance [10, 12, 34-36]. Pesticides from natural origin have generally a low toxicity and are rapidly degraded due to the low amount of halogen substituents coupled to the absence of “unnatural” rings, thus they should have a reduced impact on the environment [14, 16, 28, 34,

37-43]. When the whole extract is applied, the complex matrix contains a wide range of compounds, consequently the apparition of resistance could be delayed [14, 43, 44]. Secondary metabolites from plants and microbes were in the past regarded as waste products. However, this perception has then been revised, and these compounds are now meant to be, among others, plant natural defence mechanisms against pests (allelochemicals). Thus, they represent a great pipeline of new leads for agrochemicals and pharmaceuticals [12, 14, 37, 38, 40, 45-47]. Even the fact that plenty of natural products with pesticidal activity have been discovered, only a limited number of them have been registered and reached the market. The major reasons are [12, 14, 16, 20, 35, 36, 40, 41, 46, 48-52]:

- availability of the raw material and sustainability of its eventual cultivation
- high costs
- difficulty of standardisation
- complexity of the natural structure making the synthesis cumbersome and costly
- eventual phytotoxicity
- lack of selectivity causing toxicity to non-target organisms
- too rapid biodegradation or evaporation hindering the activity
- low or partial efficacy
- regulatory barriers
- intellectual property issues related to the Nagoya Protocol on Access to Genetic Resources and the Convention on Biological Diversity

"The Pesticide Encyclopedia", edited by Paranjape *et al*. and published in 2014 [53], includes an exhaustive list and description of natural compounds involved commercially in agriculture.

2.1.1. Fungicides and bactericides

Fungi are the most important cause of crop losses in the world and the main reasons for the essential need of fungicides are: (i) to enhance yields and diminish imperfections, (ii) to ameliorate storage life and quality of harvested plant parts, and (iii) to enhance consumer security (animals and humans). As one example of fungus which caused serious crop loss,

Cochliobolus heterostrophus Drechsler (anamorph *Bipolaris maydis* (Y. Nisik. & C. Miyake) Shoemaker), called southern corn leaf blight, damaged about 15% of all corn produced in the United States in the year 1970 leading to deficit estimated at 1 billion dollars [54]. Another severe case was the famine in Ireland triggered by potato late blight in the 1840's (See Chapter *Phytophthora infestans*). Furthermore, the security of consumers can be threatened by some fungi producing toxins [28, 55, 56]. The two most famous examples are aflatoxins and ergot alkaloids. Aflatoxins are produced principally by *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare affecting maize corn and peanuts. Once ingested, the toxins damage the liver, provoke intestinal bleeding, and cancer. The dairy products from cattle consuming these toxins are also contaminated. Ergot alkaloids are a group of toxic metabolites produced by fungi of the genus *Claviceps* that infects various plants. The most relevant pathogen is *Claviceps purpurea* (Fr.) Tul. which contaminates rye and further grasses (Figure 36). Poisoning is called ergotism and affects animals and humans. The symptoms include hallucinations, itchiness and burning feeling, loss of feet and hands, preterm birth, and even death. A disease named St. Anthony's fire in the Middle Age was characterized by the same set of symptoms and was thus attributed later to ergotism (Figure 2). Ergot alkaloids are nowadays used in medicine for various applications, such as the placenta expulsion in the third stage of labour, the control of bleeding (especially post-partum), or the treatment of migraines [55, 57].

Figure 1: Ergot of rye.
Picture: M. Viard
(HorizonFeatures/Leemage)

Figure 2: St. Anthony's Fire.
Painting: Pieter Bruegel the Elder, *The Beggars* (*The Cripples*), 1568

Several raw plant extracts are available on the market to control fungal infestation. An extract of tea tree (*Melaleuca alternifolia* (Maiden & Betche) Cheel, Myrtaceae) commercialised under the name Timorex Gold™. It seems to exert its antifungal activity by disruption of the cell membrane/wall [58-60]. Two other specialities, Biocin-T™ and BM-608™, contain the essential oil of *M. alternifolia* [4]. Another commercial product based on a crude extract is Myco-Sin™, a mixture of horsetail extract (*Equisetum arvense* L., Equisetaceae) and sulphurated clay, used against mildew, scab, rotbrenner, and fireblight [4, 61]. Plant Extract 620™ consists of four different raw extracts blended: prickly pear cactus, red mango, fragrant sumac, and Southern red oak. When applied on soils, it controls parasitic nematodes feeding on plant roots and some fungal diseases. The product is considered as safe for the environment, humans, and wildlife. However, it shows some toxicity to aquatic invertebrates, consequently its use should be avoided around water bodies [62, 63]. Finally, powder of oriental mustard seeds (*Sinapis* sp. Brassicaceae), containing the glucosinolate sinigrin (**1**) (Figure 3), is used as a fungicide and nematicide. In contact with water, the enzyme myrosinase present in the powder catalyses the hydrolytic cleavage of the glucose from sinigrin leading, after spontaneous rearrangement, to allyl isothiocyanate. Allyl isothiocyanate (**2**) (Figure 3), also called mustard oil and responsible for the pungent taste, is the pesticidal compound. Oriental mustard seed powder does not show adverse effects to non-target organisms, but allyl isothiocyanate is highly toxic to bees and mildly toxic to some other insects [63, 64].

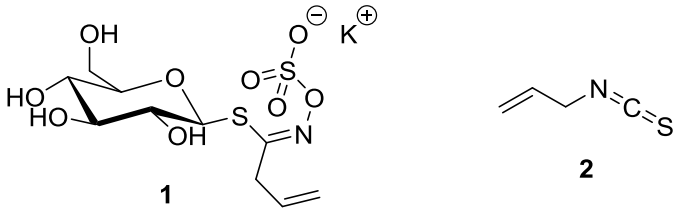

Figure 3: Sinigrin (**1**) and allyl isothiocyanate (**2**).

Some specialities are based on plant extract fractions, such as Heads Up™, a fungicide based on a mixture of saponins extracted from quinoa husks (*Chenopodium quinoa* Willd, Amaranthaceae). Quinoa is produced in large quantities in Ecuador, Bolivia, and Peru. The outer husk of the grain has a strong bitter taste and is removed before consumption. The outer husk is then a by-product and contains 20 to 30% of triterpenoid saponins. These compounds, after alkali treatment, show antifungal activity especially against *Botrytis cinerea* Pers. by disrupting the fungal cell membranes. This preparation is not expected to be deleterious to

non-target organisms, the environment, or humans, since saponins are present in several food products and degrade within three to five days [63, 65, 66]. Another product based on saponins is Quillaja Extract™, containing saponins extracted from the wood and bark of *Quillaja saponaria* Molina (Quillajaceae), the soap bark tree. It is registered for use against nematodes and fungi [63, 67]. A further example is the contact fungicide Fracture™ based on a polypeptide extract from lupine cotyledons (*Lupinus albus* L.) patented under the name BLAD™ (Banda de Lupinus albus doce). It literally fractures the cell wall by chelating effects and perturbations of the ion membrane transporters. The advantage is that there is a low risk of resistance development [58, 60]. Lecithin, a mixture of glycerophospholipids extracted from soybean (*Glycine max* (L.) Merr., Leguminosae) is applied against powdery mildew of different crops. Food-grade lecithin composes the commercial plant protection product which is thus considered as one of the safest plant-derived pesticide. The supposed mode of action of lecithin is the induction of changes in membrane permeability inducing cell lysis [68].

Some natural products with fungicidal properties are used in pure form. One of them is citric acid occurring in citrus fruits and showing a broad spectrum as contact and systemic bactericide/fungicide. This compound is commercialised as the plant protection product Procidic™ [58] and is also recommended as insecticide [69]. In addition, L-glutamic acid and γ-aminobutyric acid (GABA) are commercialised as a mixture (AuxiGro™) and used as a plant growth regulator and fungicide on a wide variety of plants. These two compounds, found in almost all living organisms, are involved in many different physiological functions. These compounds are not toxic to mammals, humans, and other organisms tested. Furthermore, since they occur naturally and degrade rapidly, their environmental profile is considered as safe [14, 69, 70].

Cinnamaldehyde is found in high quantities in the essential oil extracted from the dried bark of, for example, *Cinnamomum zeylanicum* Blume or *Cinnamomum cassia* (L.) J. Presl. (Lauraceae). This compound, usually synthesised in Vertigo™ or Cinnacure™, is used to control *Verticillium fungicola* (Preuss) Hassebr. (dry bubble) which attacks cultivated white mushrooms (*Agaricus bisporus* (J. E. Lange) Imbach) and *Fusarium moniliforme* var *subglutinans* Wollenw. & Reinking causing pitch canker disease on pine trees, or dollar spot on turfgrasses caused by *Sclerotinia homeocarpa*. By contact, cinnamaldehyde inhibits the synthesis of chitin and glucan, two constituents of the cell wall. It exerts also algaecide activity. Other uses are as attractant for corn root worms and as repellent for cats and dogs,

this being related to its strong odour. The compound presents a low risk of resistance development. Even if it can cause strong eye and skin irritation, its toxicity on mammals is low. Cinnamaldehyde degrades rapidly in the soil and is not water soluble, thus has a good ecotoxicological profile [14, 16, 36, 58, 69-72].

Certain plant extracts or compounds do not act directly as fungicides but induce the natural plant defence system, called Systemic Acquired Resistance (SAR), by raising the quantities of phenolic compounds in the tissues. The latter compounds are referred to as phytoalexins and represent defence secondary metabolites produced extemporaneously and *de novo* by the plant itself in response to a stress. Phytoalexins accumulate in the area of aggression and possess toxic or inhibitory properties against the pathogens or predators [73]. One product with this mode of action is an extract from giant knotweed (*Reynoutria sachalinensis* (F. Schmidt) Nakai, Polygonaceae). The extract, commercialised as Milsana™ or Regalia™, acts translaminarly and is recommended against a wide range of fungi and some bacterial diseases. Giant knotweed is deemed to have low toxicity to mammals and is not assumed to have any adverse effects on non-target organisms nor the environment [16, 58, 60, 69, 70, 73-78]. A further example is the aqueous extract of *Macleaya cordata* (Willd.) R. Br. (Polygonaceae), pink plume poppy, commercialised under the trade name Qwel™ against foliar fungal diseases. The two major compounds of the extract responsible for the SAR induction are the benzophenanthridine alkaloids sanguinarine and chelerythrine (**3** and **4**) (Figure 4). This extract is considered to be non-deleterious to mammals, to non-target organisms, or to the environment [16, 69, 70, 73]. In addition, Stifénia™, based on fenugreek seed extract (*Trigonella foenum-graecum* L., Leguminosae), is used against several pathogens, but especially those of grapevines. This product is classified non-toxic to humans and the environment at the recommended doses. However, its efficacy is controversial [73, 78]. Finally, *Laminara digitata* (Huds.) Lamouroux (Laminariaceae), a marine brown alga, contains a storage polysaccharide called laminarine. This polysaccharide induces plant resistance to fungal attack. The commercial product Iodus™ is recommended for cereal crops. The discovery was done by farmers in Northern France: by spreading sea weed on their fields, they realised that the plants were growing stronger and were less attacked by fungal diseases. The compound is judged unlikely to have any adverse effects on non-target organisms and on the environment. Furthermore, it is considered as safe to mammals [16, 63, 69, 70, 73, 78].

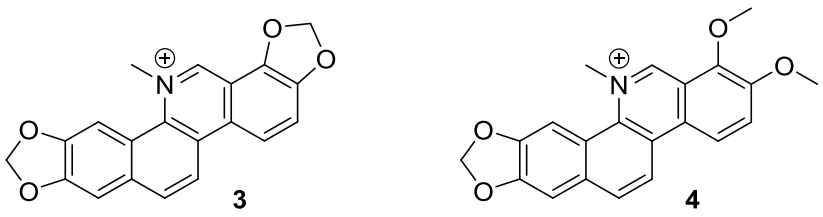

Figure 4: Sanguinarine (**3**) and chelerythrine (**4**).

Numerous bacteria, especially Actinomycetes, produce compounds with strong fungicidal and bactericidal properties, such as several *Streptomyces* species that permitted the development of potent plant protection products. The aminoglycoside streptomycin (**5**) (Figure 5) was discovered in the soil bacterium *Streptomyces griseus* (Krainsky) Waksman and Henrici. This compound, commercialised under the names Agri-Mycin 17™, Agri-Step™, Plantomycin™, or Paushamycin™, is a bactericide that acts on protein synthesis. It is systemic (xylem mobile) and is especially recommended against gram-positive bacteria. The ecotoxicological profile of streptomycin is favourable, but this compound presents a high risk of resistance, and the phenomenon is already wide spread. Therefore, it is usually applied in association with another bactericide presenting a different mode of action [16, 45, 58-60, 69]. A second example is *Streptomyces kasugaensis* Hamada *et al*. producing kasugamycin (**6**) (Figure 5), another aminoglycoside, commercialised as a protectant and curative fungicide/bactericide (Kasumin™ or Kasugamin™). This compound is also xylem mobile and inhibits protein synthesis. It presents a medium risk of resistance development, but necessitates rotation with other products. This aminoglycoside presents a really low toxicity to mammals, furthermore no adverse effects on non-target organisms and on the environment have been revealed [16, 45, 56, 58-60, 63, 69]. A further aminoglycoside is validamycin (**7**) (Figure 5) produced by the soil bacterium *Streptomyces hygroscopicus* (Jensen) Waksman & Henrici isolate *limoneus*. This antibiotic powerfully inhibits the trehalase of the phytopathogenic fungi *Rhizoctonia solani* Kühn. Trehalase is assumed to take part in the digestion of trehalose and in the distribution of glucose to the hyphs. Validamycin is a non-systemic fungistatic with a favourable ecotoxicologial profile. Commercial products are Valimun™, Mycin™, Validacin™, Vivadamy™, or Solacol™ [16, 45, 59, 60, 69].

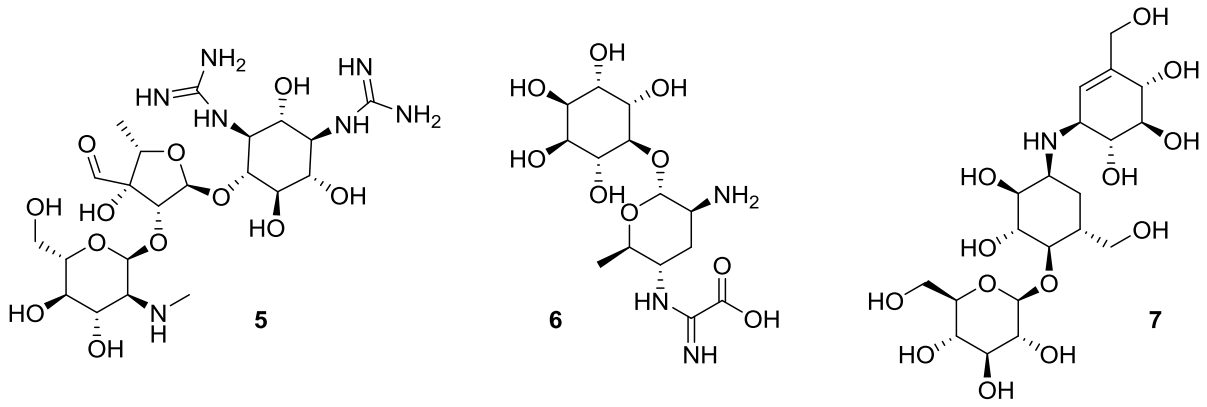

Figure 5: Streptomycin (**5**), kasugamycin (**6**), and validamycin (**7**).

Other types of antibiotics than aminoglycosides are found in *Streptomyces* species. Natamycin (also called pimaricin, myprozine, or tennectin) (**8**) (Figure 6) is a secondary metabolite of *Streptomyces chattanoogensis* Burns and Holtman and *Streptomyces natalensis* Struyk *et al.* This compound was commercialised as a fungicide for plant protection (Delvolan™), especially for the control *Fusarium oxysporum* Schlecht on ornamental bulbs (e.g. daffodils), but it has been withdrawn from the market. However, natamycin, under the trade names Natamax™ or Delvocid™, is authorised and used as a food preservative. It exerts its activity by binding to ergosterol, an essential constituent of fungal cell membranes, leading to membrane dysfunction. The compound is relatively non-toxic to mammals, and no adverse effects to non-target organisms or to the environment have been noticed [16, 45, 69, 79]. A further antibiotic type is polyoxins (**9**) (Figure 6) produced by *Streptomyces* spp. such as *Streptomyces cacaoi* Waksman and Henrici. These compounds are protective systemic antifungal by inhibiting the chitin synthase on a single site, but have a limited spectrum of action (ineffective against yeasts and bacteria). For example, polyoxin D is commercialised as Endorse™, Oso™, or Ph-D™. Polyoxins have very low mammal toxicity and have not exhibited unfavourable effects on the environment and non-target organisms [45, 58-60, 63, 69].

Blasticidin S (**10**) (Figure 6) isolated from the soil bacterium *Streptomyces griseochromogenes* Fukunaga is commercialised in different formulations under the name Bla-S™ and is used to control rice blast (*Pyricularia oryzae* Cavara; perfect stage *Magnaporthe grisea* (Hebert) Barr). However, applied in too high amounts, it can show phytotoxicity. This contact bactericide/fungicide is protective and curative by inhibiting protein biosynthesis. Even if it presents some toxicity to mammals (acute oral LD_{50} to rodents

is lower than 100 mg/kg) and severe irritant effect on the eyes, its environmental profile is fair and the product is comparatively non-toxic to non-target organisms [16, 45, 56, 59, 60, 69]. In addition, oxytetracycline (**11**) (Figure 6), a tetracyclic antibiotic, is produced by the bacterium *Streptomyces rimosus* Sobin *et al.* This antibiotic (FireLine™, Mycoshield™, or Terramycin™) is truly systemic (amphi-mobile), meaning it moves upwards with the xylem and downwards with the phloem. Its mechanism of action is the disturbance of protein synthesis, and presents a high risk of resistance development. Oxytetracycline controls fireblight (*Erwinia amylovora* (Burrill) Winslow) and diseases triggered by *Xanthomonas* spp and *Pseudomonas* spp. The antibiotic is considered as non-toxic to mammals and displays no unfavourable effects on the environment and on non-target organisms [16, 45, 58-60, 69]. Finally, the soil bacterium *Streptoverticillium rimofaciens*, another type of Actinomycete, generates mildiomycin (**12**) (Figure 6). This compound specifically controls powdery mildews. Mildiomycin™ presents some systemic activity and acts as an eradicant. Its mode of action is supposed to be through the inhibition of protein synthesis by a blockage of peptidyl-transferase. The compound is not toxic to mammals and presents a good ecotoxicological profile [16, 45, 69].

Two *Pseudomonas* species produce interesting compounds. Pyrrolnitrin (**13**) (Figure 6) from *Pseudomonas pyrrocinia* Imanaka *et al.* led to the development of two synthetic pyrroles: fenpiclonil and fludioxonil (**14** and **15**) (Figure 6). Fenpiclonil is no longer on the market. Fludioxonil (Celest™) is used as seed-dressing against fungal pathogens and as foliar fungicide against *B. cinerea*, *Monilinia* spp., and *Sclerotinia* spp. Their mechanism of action is supposed to be glucose metabolism inhibition by targeting a transmembrane sugar carrier [34, 56, 59, 60]. Rhamnolipids found in *Pseudomonas aeruginosa* Schröter act as biosurfactants and destroy the cell membranes of fungal zoospores. The commercial fungicide Zonix™ contains a mixture of rhamnolipids 1 and 2 (**16** and **17**) (Figure 6). These glycolipids are non-toxic, non-mutagenic, and easily degraded [4, 40, 58, 63, 80].

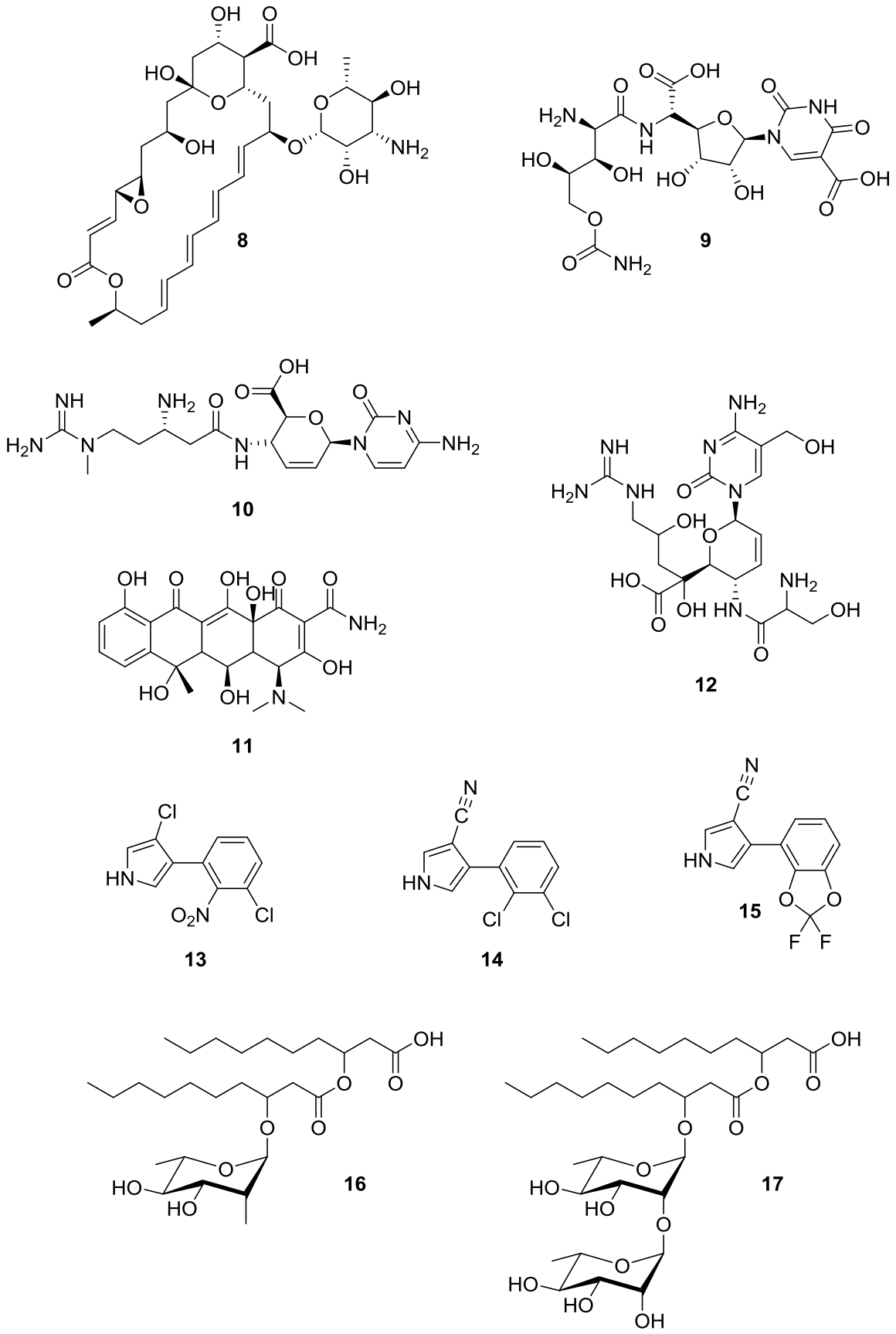

Figure 6: Natamycin (**8**), polyoxin D (**9**), blasticidin S (**10**), oxytetracycline (**11**), mildiomycin (**12**), pyrrolnitrin (**13**), fenpiclonil (**14**), fludioxonil (**15**), rhamnolipid 1 (**16**), and rhamnolipid 2 (**17**).

Two Basidiomycetes fungi growing on decaying wood, *Strobilurus tenacellus* (Pers.) Singer and *Oudemansiella mucida* (Schrad.) Hoehn., are producing strobilurins, one of the most important class of agricultural fungicides. The natural products are volatile and highly photosensitive, inducing a too rapid degradation. Arising from that, they are not usable without chemical modifications for stabilisation. Azoxystrobin (**18)** (*β*-methoxyacrylate) was synthesised from strobilurin A (**19**) (Figure 7) and is available under different names such as Amistar™, Abound™, Heritage™, Stadium™, or Quadris™. This compound possesses a strong antifungal activity with a broad spectrum. In addition, it has a low toxicity on mammalians and a good environmental profile. The antifungal activity of strobilurins is based on the inhibition of the mitochondrial respiration (halting ATP production). They bind to the Q_o-site (ubiquinol oxidation-site) of cytochrome b, part of Complex III located in the inner mitochondrial membrane. In 1999, after four years of commercialisation, the sales of azoxystrobin reached 415 million dollars. In 2017, the compound was still the most sold fungicide in the world. In addition to azoxystrobin, four other derivatives of strobilurins were on the U. S. market in 2016: kresoxim-methyl (Cygnus™ or Sovran™), fluoxastrobin (Disarm™ or Evito™), trifloxystrobin (Compass™, Flint™, or Gem™ 500SC), and pyraclostrobin (Cabrio™, Headline™, Insignia™, or Priaxor™). They are partially absorbed in plant tissues (translaminar or xylem mobile, depending on the derivative) and exert preventive/curative properties by inhibiting sporulation, spore germination, and mycelial growth. These fungicides are site-specific, so highly prone to development of resistance. There is a growing number of pathogens that are reported as resistant worldwide [16, 36, 37, 40, 42, 51, 56, 58-60, 63, 81-83].

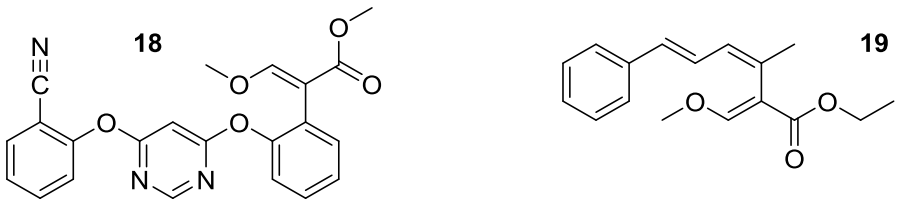

Figure 7: Azoxystrobin (**18**) and strobilurin A (**19**).

Chitin is a polysaccharide (poly-N-acetyl-*D*-glucosamine) present in the shell of all insects, crustaceans, and in some other organisms, such as algae, fungi, and yeast. This polymer is applied on soils to control plant pathogenic nematodes. The postulated mode of action is the increase of some soil microorganisms which produce toxins that kill the nematodes and their eggs. Commercially, chitin is extracted from crustacean shells and a

traded plant protection product is Clandosan™ [69, 84]. Additionally, chitosan, the deacetylated derivative of chitin, is also utilised as a plant protection product (trade names ChitoPlant™ or Elexa™) against bacteria and fungi, especially powdery mildews and *Botrytis* species. The polymer also has an application post-harvest; it reduces the decay and delays the ripening of fruits and vegetables. Chitosan mimics an attack by fungal spores and stimulates resistance by sending signals to the plant cell nuclei. This triggers the production of phytoalexins in addition with other genetic and biological responses. The ecotoxicological profile of these polymers is excellent [4, 16, 34, 69, 73, 78, 84, 85].

2.1.2. Insecticides and acaricides

For thousands of years, insects have been causing dissemination of calamitous infectious diseases to humans and animals, havoc of crops and food reserves, deterioration of forests, and disturbance of the ecosystem. They decrease agricultural yields by 10 to 16% before harvest and about the same quantity is devoured post-harvest [86]. Two examples that are particularly remembered in Europe are Colorado beetles and phylloxera. Colorado beetles, *Leptinotarsa decemlineata* Say, mainly attack potatoes, tomatoes, and aubergines (Figure 8). They were first noticed in Colorado in 1811, then arrived in France in 1922, and spread throughout Europe rapidly. Without appropriate control, major to complete destruction of the crop occurs. Colorado beetles were even used during Second World War to bomb the enemy crop fields [87]. The second example, phylloxera (*Daktulosphaira vitifoliae* Fitch), a small sap sucking insect, is a terrible scourge destroying grapevines (Figure 9). The symptoms were first observed in 1866 where 5 hectares were destroyed in a lower Rhône French vineyard. Then, in only some of the years following, it spread all over the major European wine producing areas. The best actual solution to fight against phylloxera is to graft resisting rootstocks [88].

In agriculture, the use of plants as insecticides is known since at least two millennia in Greece, India, Egypt, and China. With the arrival of major classes of synthetic products in the mid-1930s to 1950s, botanicals fell into oblivion. Nevertheless, the overuse of synthetic insecticides led to many severe problems, such as acute or chronic human poisoning, large groundwater contamination, apparition of resistances, and damage to birds, fish, pollinators, and further wildlife. As a consequence, governments rigorously restricted their use and implemented policies to replace these chemicals by safer alternatives in the early 1990s. This led to a renewed interest for plant secondary metabolites [35, 44, 49]. Nowadays, several

products derived from natural products are commercially used as insecticides. However, some limitations are slowing down their development, including short residual activity, mostly limited contact activity (ingestion necessary), effects predominantly on immature insects (precise timing required), no systemic activity, short shelf life, and lack of efficacy data [89].

Figure 8: Colorado beetles on potato plant. Picture: D. Cappaert (www.insectimages.org)

Figure 9: "The phylloxera, a true gourmet, finds out the best vineyards and attaches itself to the best wines." Cartoon: Punch (The London Charivari), 6th September 1890

One example of natural non-systemic insecticide and acaricide by contact is Requiem™ (formerly Facin™ or Keynote™) based on the extract of *Chenopodium ambrosioides* L. near *ambrosioides* (Amaranthaceae), the American wormseed, containing mainly terpenoids. Its modes of action on insects are trachea collapse causing asphyxia, disruption of the cuticle leading to desiccation, and feeding deterrent properties. Thrips, mites, white flies, and aphids are controlled by Requiem™. The leaves are part of traditional Mexican cooking. This plant was also used during late 19th and early 20th as nematicidal drug for humans and livestock. The extract is considered not significantly deleterious to humans or the environment and degrades rapidly [44, 63, 90, 91]. Another insecticide is starch syrup, obtained mainly from potatoes and corn, commercialised under the name YE-621™. Its activity is explained by the obstruction of insect spiracles and its advantage is efficacy towards insects resistant to chemical pesticides. Additionally, it is non-toxic to humans and to beneficial insects [35, 69].

Formic acid is used to control *Varroa destructor* Anderson & Trueman and tracheal mites of honey bees. Formic acid is an acute irritant acting on the mites without having considerable effects on bees. To fight against varroa, it is also possible to use Apilife VAR™, a mixture of thymol, eucalyptus oil, *L*-menthol, and camphor [46, 63, 69]. Capsaicin, the compound responsible for the hotness of chilli peppers from the genus *Capsicum* (Solanaceae), is claimed to possess a wide range of properties, including animal repellent, feeding depressant, insecticide, fungicide, miticide, molluscicide, nematicide, rodenticide, and herbicide. The repellent effect is explained by the pungent odour and taste coupled to irritation. The pesticide effect is considered to be due to metabolism disruption, affection of the central nervous system, and impairment of cell membranes. Commercial products include Valoram™, Armorex™, Nemastroy™, or Dazitol™. Acute oral LD_{50} values were determined in female and male mice as 97.4 and 118.8 mg/kg respectively, and in female and male rats as 148.1 and 161.2 mg/kg, respectively. In the environment, capsaicin is rapidly degraded, but it is deleterious to beneficial insects, including honey bees [12, 16, 36, 53, 69].

Some natural compounds have specific modes of action to exert their insecticide activities. This is the case of karanjin and azadirachtin interfering with ecdysteroids hormones, thus acting as an insect growth regulator. Karanjin (**20**) (Figure 10) is a furanoflavonol found in the seeds of *Pongamia pinnata* (L.) Pierre (Leguminosae) called Indian beech, pongam, or karanj and growing in the south of India. The molecule is an acaricide and insecticide which acts by blocking ecdysteroids effects, and on some organisms, it additionally inhibits cytochromes P-450. Furthermore, strong anti-feeding property is wielded. Some fungicidal properties are also reported. The plant extract is commercialised under the name Derisom™. No allergic or adverse effects were reported on users, and the compound is not likely to have any adverse effects on non-target organisms and on the environment [12, 14, 35, 69]. The limonoid tetranortriperpenoid azadirachtin (**21**) (Figure 10) is the main systemic active compound of the seed extracts of *Azadirachta indica* A. Juss. (Meliaceae), called neem tree, growing widely in India. The observation was made that this tree was not susceptible to insect attacks. Many formulations containing pure azadirachtin or neem oil are commercialised (Neemix™, Aza-Direct™, Triact™, or Azatin™). Azadirachtin blocks the synthesis of ecdysteroids hormones and their release. Neem products also have strong anti-feeding and repellent effects. In addition, it has been shown that azadirachtin inhibits microtubule formation at the cellular level. The spectrum of activity is broad (whitefly, leaf miners, thrips, caterpillars, aphids, beetles, jassids, and

maelybugs). Azadirachtin also possesses antifungal properties. Thus, the speciality Trilogy™ is recommended as a miticide, insecticide, and fungicide. Nematicidal properties were also reported. The compound, rapidly degraded or isomerised by exposure to sunlight, is relatively non-toxic to mammals (acute oral LD_{50} on rats of >3540 mg/kg), to pollinators, and fish. It is not mutagenic, as well as not irritant to skin and eyes. However, its impact to beneficial insects is highly variable, and should be taken carefully into consideration [12, 14, 16, 20, 31, 35, 36, 40-43, 45, 46, 49, 58-60, 69, 78, 89, 92-98]. A semi-synthetic derivative of azadirachtin was developed consisting of a hydrogenated form, the dihydroazadirachtin, commercialised under the name DAZA™. The range and mechanisms of action as well as its toxicity are highly similar with those of the natural compound [35, 69].

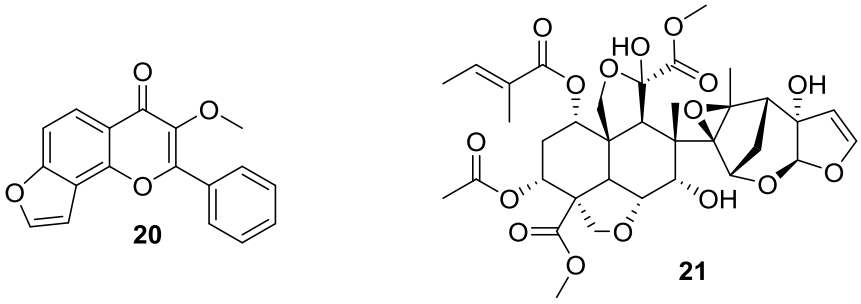

Figure 10: Karanjin (**20**) and azadirachtin (**21**).

Another mode of action is specific interaction with ion channels. The ryanodine receptors are calcium channels located in the endoplasmic reticulum of heart and skeletal muscle cells of insects. The alkaloid ryanodine (**22**) (Figure 11) and analogues, found in the Salicaceae *Ryania speciosa* Vahl (Caribbean shrub) stems, bind to the open form of these receptors. The binding of ryanodine triggers the discharge of calcium, leading to intense muscular contractions, and fast occurring death. Ryanodine is a non-systemic insecticide mainly used to control codling moth and citrus thrips (Ryan 50™ or Natur-Gro Triple Plus™). It shows moderate mammalian toxicity (LD_{50} between 750 to 4000 mg/kg) and high toxicity to fish since ryanodine is not selective for insect receptors [12, 14, 16, 20, 31, 35, 42, 43, 45, 49, 59, 60, 69, 95, 96, 98].

The powdered dry flowers of *Tanacetum cinerariifolium* (Trevir.) Sch. Bip. (Asteraceae) have been used as an insecticide since the ancient times in China and probably spread West through the Silk Routes during the Middle Ages. The material was commonly named "Persian Insect Powder". The secondary metabolites responsible for the insecticidal and acaricidal activity are pyrethrins with the two most abundant being pyrethrin I and pyrethrin

II (**23** and **24**) (Figure 11), followed by cinerin I, cinerin II, jasmolin I, and jasmolin II. This non-systemic contact pesticide has a broad spectrum and exerts neurotoxic activity by blocking voltage-dependent sodium channels of nerves, heart, and skeletal muscles, causing paralysis before death. The products have a really short residual activity (high instability to air, light, and moisture), but are effective rapidly. Pyrethrins have a relatively low toxicity to mammals (rat oral acute LD_{50} between 350 to 500 mg/kg) and to birds, although they are highly toxic to fish, aquatic invertebrates, and honey bees. Pyrethrins are often commercialised in mixtures with other compounds, such as with piperonyl butoxide (**25**) (Figure 11), a synergist slowing down the detoxification within the target organism. Different examples are PyGanic™, Evergreen™, Pyrocide™, or ExciteR™. A combination of pyrethrins and azadirachtin is also available (Azera™). Natural pyrethrins were used as a scaffold for a wide range of synthetic derivatives, called pyrethroids, with optimised properties and stability such as permethrin (Ambush™ or Pounce™) or deltamethrin (Decis™) (**26**) (Figure 11) [12, 14, 16, 20, 31, 35, 36, 40-43, 45, 46, 49, 59, 60, 63, 68, 69, 89, 95, 96, 98, 99].

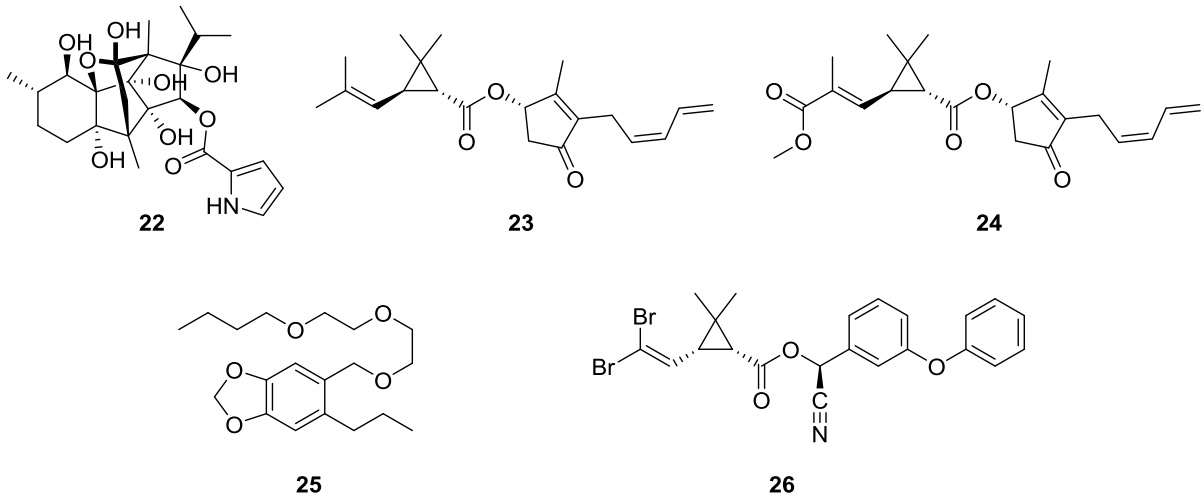

Figure 11: Ryanodine (**22**), pyrethrin I (**23**), pyrethrin II (**24**), piperonyl butoxide (**25**), and deltamethrin (**26**).

The seeds of sabadilla (*Schoenocaulon officinale* (Schltdl. & Cham.) A. Gray, (Melanthiaceae) are a non-systemic insecticide after contact and ingestion against a wide range of insects, except aphids and spider mites. They contain mainly a mixture of cevadine and veratridine (2:1) (**27** and **28**) (Figure 12). These compounds act similarly to the pyrethrins on voltage-dependant sodium channels, but apparently on a different binding site.

Crushed seeds of sabadilla were used as an insecticide by native people of South and Central America. The persistence of veratridine is longer than cevadine, however they are both degraded by air and sunlight. Seven days after application, no residual activity is detected. Pure cevadine is highly toxic with a rat oral LD_{50} of circa 13 mg/kg. However, the commercial products (Red Devil™ or Natural Guard™) contain less than 1% of active compounds, giving a safety margin. Sabadilla shows low toxicity to mammalians and to non-target organisms, but is toxic to bees. Nevertheless, it is irritant to mucosa causing sneezing and to eyes triggering inflammation and lacrimation [12, 14, 16, 20, 31, 35, 36, 43, 45, 46, 49, 59, 60, 69, 95, 96, 98].

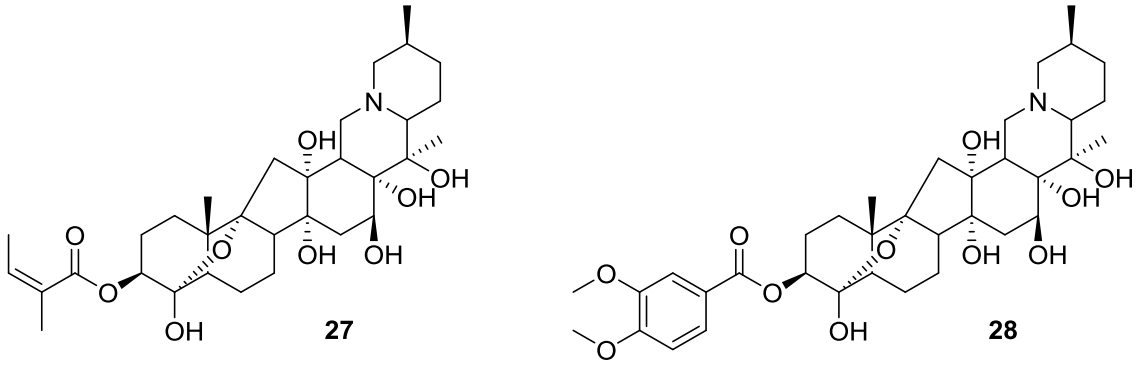

Figure 12: Cevadine (**27**) and veratridine (**28**).

Avermectins and milbemycins possess similar scaffolds and exert their insecticidal activity by interfering with neuromuscular and neural transmission through interaction with GABA- and glutamate-gated chloride channels by contact or after ingestion. Avermectins are fermentation products of the soil bacterium *Streptomyces avermitilis* (ex Burg et al.) Kim and Goodfellow. They were discovered in the course of a screening for anthelmintic natural products. These compounds possess also acaricidal properties and show translaminar distribution. Abamectin, also called avermectin B1, is a mixture of avermectin B_{1a} (>80%) and avermectin B_{1b} (<20%) (**29** and **30**) (Figure 13) commercialised under different trade names such as Dynamec™, Agri-Mek™, Avid™, Affirm™, Abacid™, or Zephyr™. Avermectins show high acute oral toxicity to rats and mice (LD_{50} of 10.0 and 13.6 mg/kg, respectively). However, abamectin has low toxicity against non-target organisms due to the low amounts used, its low water solubility, and tight binding to soils. These properties also avoid the trickling in groundwater or the contamination of the aquatic environment. In addition, it is rapidly degraded by exposure to light and by soil microorganisms, therefore it does not accumulate in the environment [36, 37, 40, 42, 45, 59, 60, 92, 95, 100, 101]. Close

semi-synthetic analogues of avermectins B_{1a} and B_{1b} (4”-deoxy-4”-methylamino derivatives) called emamectins benzoates B_{1a} and B_{1b} were developed. These compounds are also commercialised in a mixture as insecticide and acaricide (Affirm™, Proclaim™, Arise™, Shot-One™, or Denim™). Their efficiency is higher on Lepidopteran after ingestion and their toxicity against mammals is lower than that of avermectins (oral acute LD_{50} to rats of 70 mg/kg). However, they are highly deleterious to beneficial insects (especially honey bees), so that sprays should be avoided during flowering periods [16, 42, 45, 51, 59, 60, 69, 96].

Milbemycins, are secondary metabolites produced by the soil bacterium *Streptomyces hygroscopicus* (Jensen) Waksman & Henrici subsp. *aureolacrimosus* and have a narrower spectrum than abamectin. The composition of commercial products, such as Milbeknock™, Koromite™, or Matsuguard™, is described as milbemectin, consisting of a mixture of ≤30% milbemycin A_3 and ≥70% milbemycin A_4 and (**31** and **32**) (Figure 13). It shows moderate oral toxicity to mammals, does not remain in the environment, and is relatively non-toxic to non-target organisms, even though it affects some beneficial insects [16, 36, 45, 59, 60, 69, 95].

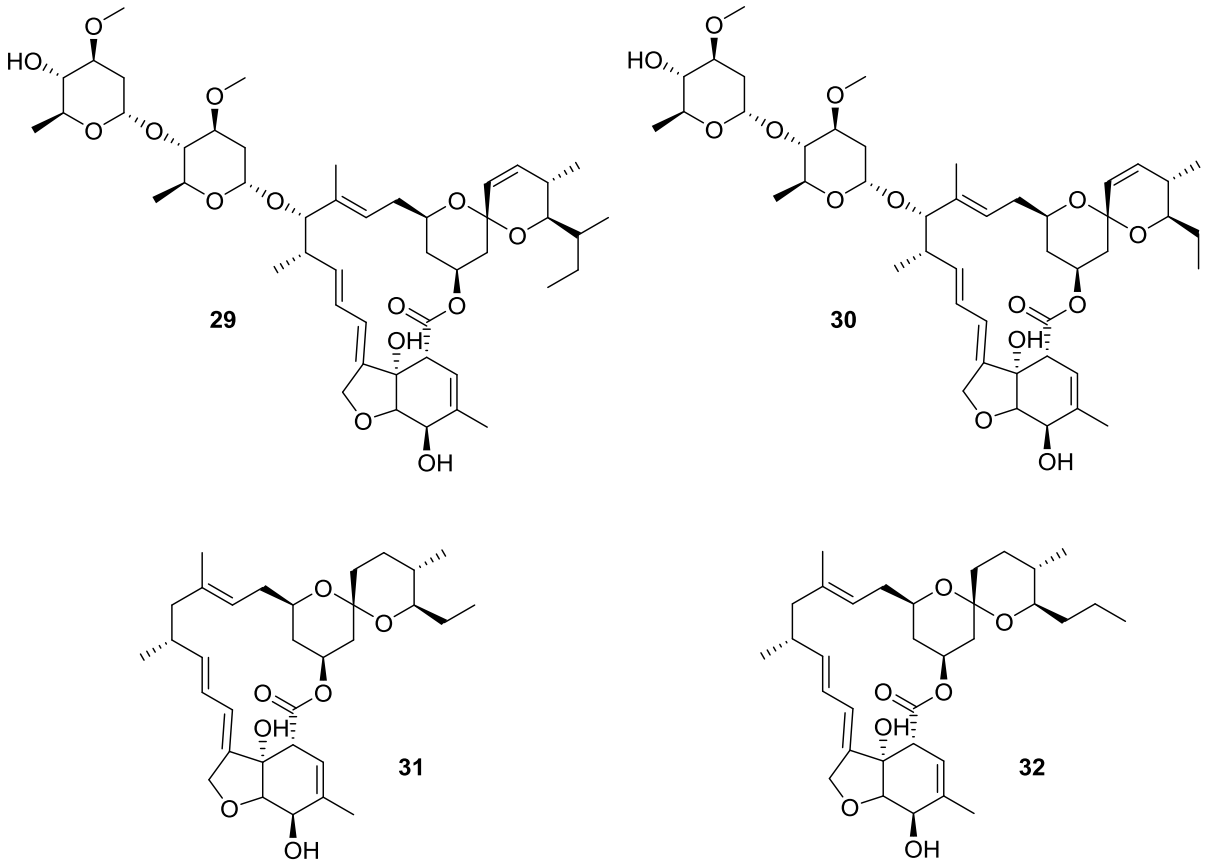

Figure 13: Avermectin B_1a (**29**), avermectin B_1b (**30**), milbemycin A3 (**31**), and milbemycin A4 (**32**).

The use of tobacco aqueous extract as an insecticide was recorded in 1690. The alkaloid nicotine (**33**) (Figure 14) is responsible for the activity and is a major compound in *Nicotiana* spp. (Solanaceae), especially in *N. rustica* L. Nicotine is non-systemic and mimics acetylcholine by binding to the nicotinic acetylcholine receptor (NAChR) in the insect nervous cells, resulting in a continuous receptor firing. Its activity is the highest in vapour phase, but it is also slightly efficient by contact or after ingestion. This alkaloid is used against a broad range of insects to protect ornamentals and crops (Tobacco Dust™, XL-All Nicotine™, Black Leaf 40™, or Nicotine 40% Shreds™). (*L*)-Nicotine is two to three times more active than (*D*)-nicotine. The alkaloid is highly toxic to humans and other mammals by inhalation and skin contact (rat oral LD_{50} of 50 mg/kg). In addition, it is deleterious to birds, fish, aquatic organisms, and bees [12, 14, 16, 20, 31, 36, 41, 43, 45, 46, 49, 63, 69, 95, 96, 98]. Semi-synthetic modifications of nicotine, mainly to reduce its toxicity, led to neonicotinoids, the actual major class of systemic insecticides with low acute and chronic toxicity to mammals, birds, and fish, but which still present high deleterious effects on bees. Examples of neonicotinoids are imidacloprid (**34**) (Figure 14) (Admire™, Confidor™, Merit™, or Provado™), acetamiprid (**35**) (Figure 14) (Mospilan™), or thiamethoxam (Actara™ or Platinum™). The selectivity for insects versus mammals is explained by the fact that neonicotinoids are not protonated at physiological pH, in contrast to nicotine which is cationic. The protonated site is needed for binding with mammal NAChR. This cationic site is replaced in neonicotinoids by an electronegative tip involving a nitro or a cyano group, supposed to lead to the binding to specific cationic subsite of the insect NAChR [12, 29, 41, 59, 63, 95, 96, 102]. Like nicotine, spinosyns also act on NAChR and provoke hyperexcitation of the nervous system, but bind on a different site of the receptor. Spinosyns are macrocyclic lactones found in the Actinomycete soil bacterium *Saccharopolyspora spinosa* Mertz & Yao. This bacterium was isolated from soil collected around a sugar mill of an abandoned rum still in the Caribbean. A mixture of spinosyn A (85%) and spinosyn D (15%) (**36** and **37**) (Figure 14), named spinosad, is commercialised as Entrust™ Naturalyte™, Tracer™, SpinTor™, Success™, or Conserve™. Spinosad is an insecticide by contact or ingestion with a high efficacy and broad range of activity (thrips, fleas, Lepidopterans, Dipterans, and Hymenopterans). Spinosyns show very low toxicity to mammalians and non-target organisms and are non-toxic to birds. However, they are slightly to moderately toxic to fish and highly toxic to bees. Nevertheless, these compounds are rapidly decomposed by light exposure on the surface and in the soils by microorganisms [16, 36, 37, 40, 42, 45, 59, 60, 63, 68, 69, 89, 95, 96, 103]. Subsequently, semi-synthetic

derivatives were developed from the mixture of spinosyns J and L resulting in spinetoram. Spinetoram, commercialised under the trade names Delegate™, Radiant™, or Exalt™, has a higher effectiveness especially against whiteflies and a longer residual activity than natural spinosyns. It also shows a good safety profile to mammals and the environment [42, 51, 59, 60, 104].

Pyripyropenes produced by the Ascomycete *Aspergillus fumigatus* Fresenius are known to activate the vanilloid-type transient receptor potential (TRPV) channels, which lead to disturbance of motor coordination and feeding capacity. These channels are expressed only in insect chordotonal stretch receptor neurones. Pyripyropene A (**38**) (Figure 14) shows strong insecticidal properties and this led to the development of the semi-synthetic derivative afidopyropen (**39**) (Figure 14), active against piercing and sucking insects. The regulatory dossier was submitted in 2016 [42, 59, 60, 105, 106].

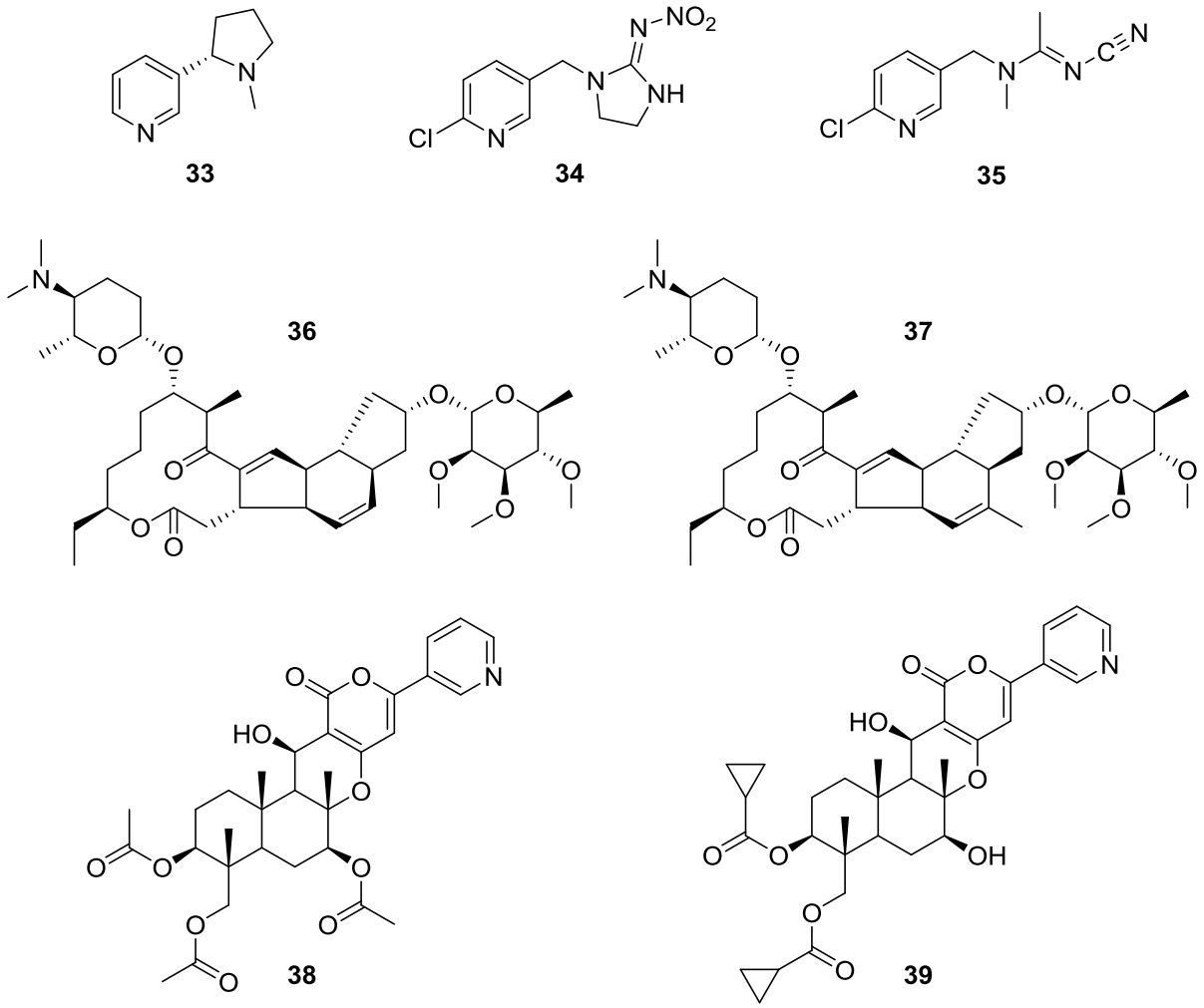

Figure 14: Nicotine (**33**), imidacloprid (**34**), acetamiprid (**35**), spinosyn A (**36**), spinosyn D (**37**), pyripyropene A (**38**), and afidopyropen (**39**).

The following compounds act in the mitochondria and block the energy production of the cell. Rotenone (**40**) (Figure 15), extracted from the Leguminosae species *Derris* spp., *Lonchocarpus* spp., and *Tephrosia* spp., is a selective/non-systemic insecticide and acaricide used for more than 150 years against a large range of arthropods in vegetable and fruit crops. In addition, it can be utilised to control fire ants and mosquito larvae in ponds. Rotenone is effective after ingestion and contact by blocking the electron transport chain in the mitochondria. The compound is judged as moderately toxic to mammals by injection (acute LD_{50} to rats of 132 mg/kg), but inoffensive by ingestion. It is highly toxic against fish (first use was as fish poison), cold blood animals, and non-target insects. The compound is rapidly degraded in the environment with a DT_{50} of 5 to 7 h under field conditions (DT = Dissipation Time). The safety of rotenone has been questioned with studies on rats showing the development of brain lesions after exposure. The lesions were similar to those observed in humans and animals suffering from Parkinson's disease. Furthermore, residues of the compound are found in the food crop after harvest which was especially demonstrated in olives and olive oil. Thus, rotenone is likely to be withdrawn from the market, because it was not included in the re-evaluation process for old pesticides in the European Union [12, 14, 16, 20, 31, 35, 36, 41, 43, 46, 49, 68, 69, 95, 96, 98].

The synthetic naphthoquinone derivative acequinocyl (**41**) (Figure 15) is utilised commercially as an acaricide (Kanemite™). It controls numerous species of mites in all growth stages, and does not have deleterious effects on beneficial predatory mites. Furthermore, acequinocyl has a short environmental persistence with a DT_{50} of 3 days and has a low toxicity to mammals with a LD_{50} to rats of 5000 mg/kg. This compound is a proacaricide. After deacetylation by hydrolysis, it binds to the ubiquinol oxidation-site (Q_o-site) of Complex III and blocks mitochondrial respiration, similarly as the stobilurin fungicides aforementioned in Chapter 2.1.1 [46, 63, 107, 108].

Streptomyces fumanus Pridham produces different pyrrolomycins. The major component dioxapyrrolomycin (**42**) (Figure 15) is a potent uncoupler of oxidative phosphorylation in the mitochondria and shows moderate activity against some insects and mites. This led to the development of the derivative chlorfenapyr (**43**) (Figure 15), commercialised under the names Pirate™, Stalker™, Phantom™, or Pylon™. Chlorfenapyr is also a propesticide activated through the removal of the N-ethoxymethyl group by oxidation. After contact or ingestion, this metabolite disrupts the ATP production by acting on oxidative phosphorylation in the mitochondria, as aforementioned for the natural product dioxapyrrolomycin. The

compound also shows inhibitory properties on chitin biosynthesis. Chlorfenapyr controls a wide range of insects and phytophagous mites, including some resistant to other pesticides. It is insoluble in water and distributes translaminarly in the plant, but has a limited systemic activity. The compound is considered as moderately toxic, with an acute oral LD_{50} of 441 mg/kg in male rats and of 1152 mg/kg in female rats, but has an extended environmental persistence (DT_{50} > 365 d). For one bee, the LD_{50} is 0.2 mg. Furthermore, it has some deleterious effects on bird reproduction. Chlorfenapyr is non-irritant to the skin, but moderately to the eyes [53, 59, 60, 63, 95, 108-112]. Finally, *Streptomyces aureus* Manfio *et al*. produces polynactins that are mainly a mixture of three different compounds: tetranactin (**44**) (Figure 15), trinactin, and dinactin. These compounds, in wet conditions, are very efficient to control spider mites and the European red mite on fruit trees. The postulated mode of action is the leakage induction of cations, for example potassium ions, through the mitochondrial membrane. The compounds are considered non-poisonous to mammals and relatively non-toxic to beneficial insects, but they are highly noxious to fish. To reduce the risks of increased resistance, polynactins are commonly sold in combination with other acaricides, such as fenobucarb (Mitecidin™) or febutatin oxide (Mitedown™) [45, 69].

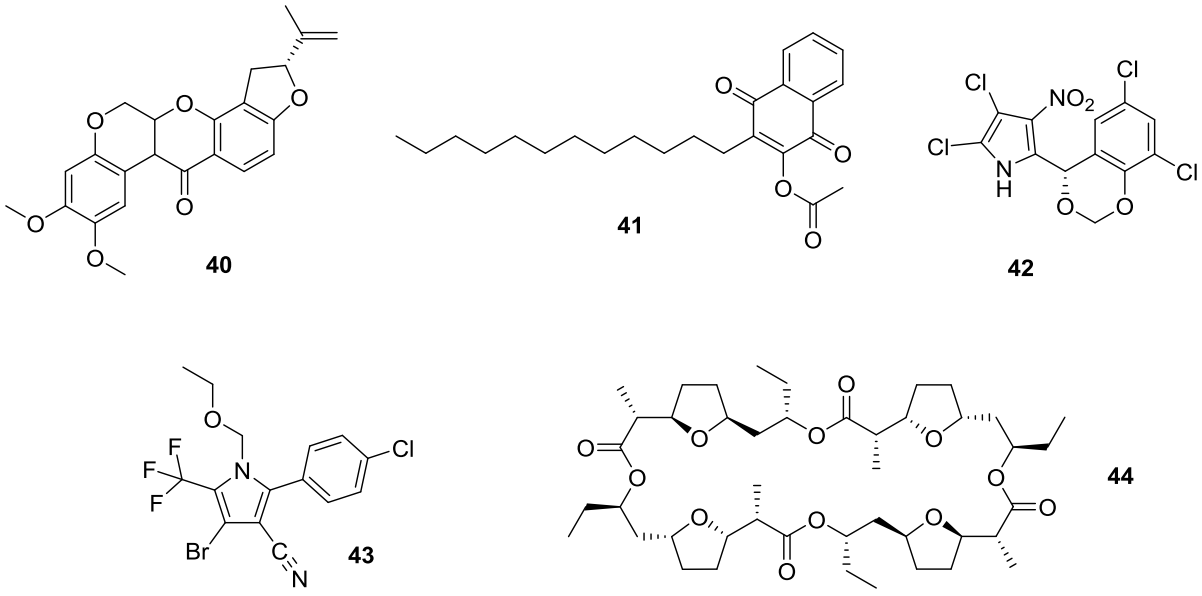

Figure 15: Rotenone (**40**), acequinocyl (**41**), dioxapyrrolomycin (**42**), chlorfenapyr (**43**), tetranactin (**44**).

An enthralling new pesticide for controlling thrips and whiteflies in greenhouses reached the market in 2018 under the trademark Spear T™. This contact insecticide is based on a spider toxin, GS-omega/kappa-Hxtx-Hv1a, which acts as a neurotoxin. The omega-Hxtx-

Hv1a peptide antagonises the voltage-gated calcium channels and the kappa-Hxtx-Hv1a, the voltage-gated potassium channels. Since the diet of most spiders is based on different invertebrates, especially insects, they developed a collection of products that act on a broad spectrum of species. Very few of these toxins are deleterious to humans and other mammals, even if some large spiders feed on small vertebrates. Since the peptides have a disulfide-rich molecular architecture, they are relatively stable, especially towards proteases, thereby enhancing residence time in the insect body and persistence of the activity on treated fields. In addition, they are not expected to release toxic residues after degradation [42, 113-115].

Finally, the bacterium *Bacillus thurigiensis* Berliner (Bt) produces crystalline, proteinaceous inclusions on its spores that are toxic after ingestion to Lepidopteran larvae, to some Coleopteran adults and larvae, and to some Dipteran larvae. This agent has been one of the most studied and commercially used bacterial biocide in the last 40 years. Various products containing spores are available on the market, such as Thuricide™ or Monterey B.t.™. The mode of action of this toxin is described on Figure 16. The strengths of this toxin are its safety for human beings and its inoffensive effect on the natural Lepidopteran predators. However some resistance is emerging in some pests, so that research for novel strains and toxins with novel modes of action is strongly needed [31, 42, 45, 59, 60, 89, 116, 117].

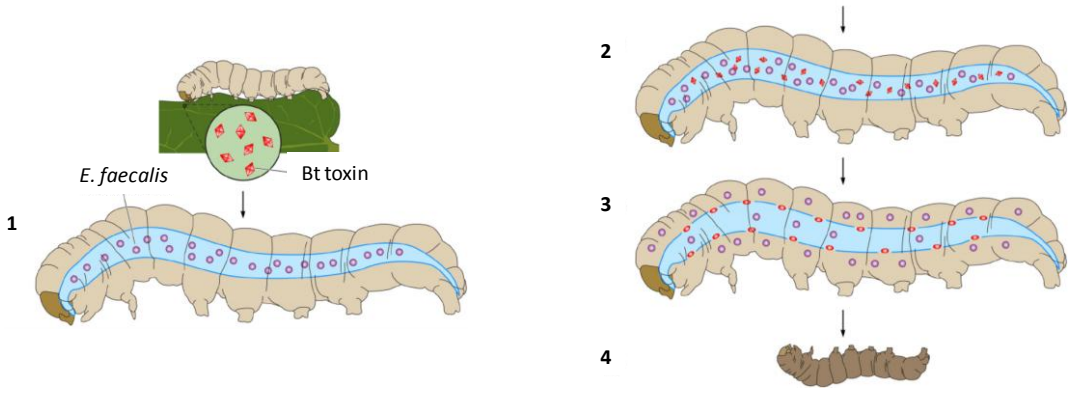

Figure 16: *Bacillus thurigiensis* (Bt) toxin mode of action on caterpillar: *B. thurigiensis*, during sporulation, produces Bt toxin, a *δ*-endotoxin, that forms proteinaceaous crystal inclusions on the spore surface. The spores are then ingested by the caterpillar (**1**) and reach the midgut colonised by *Enterococcus faecalis* (**2**) (purplish cocci on the cross-section). The ingestion leads to the germination of the spores and the alkaline environment of the digestive tube releases the inclusion, thus solubilising the protoxin (red diamonds). After the protoxin has been activated by the proteases of the insect, it binds to the gut wall and creates pores. Through those pores, *E. faecalis* migrates to enter the hemolymph (**3**) leading to death by septicaemia (**4**). Adapted from [116]

2.1.3. Herbicides

Herbicides are used to fight against weeds. A weed is defined in different ways, but the most common are: (i) “a plant that is out of place and not intentionally sown”, (ii) “a plant that grows where it is not wanted or welcomed”, or (iii) “a plant that is competitive, persistent, pernicious, and interferes negatively with human activities”[118]. Among other problems, the main issue is that they reduce crop yield by competition for soil nutrients, space, water, light, and CO_2 [118]. Nowadays, resistance to commercial herbicides is evolving rapidly, and over the last 20 years no product with a new mode of action has been introduced to the market. Furthermore, some products were withdrawn due to safety issues. Therefore, the need for innovation in this field is imperative. Herbicides based on natural products are considered safer than synthetic compounds and are a great pipeline for discovery of novel modes of action [42, 119].

Only a handful of natural products are used commercially as herbicides. One example is aqueous vinegar sprays containing up to 20% of acetic acid used for non-selective weed management on non-crop land areas (for example on railways, driveways, industrial sites, or golf courses). This product acts by burning the top of the weed, but does not affect the root system. Its efficacy is higher on broadleaf than on grassy weeds. However, regularly repeated sprayings are necessary, since the plants may recover from the foliar damages. Acetic acid is sometimes mixed with other plant-derived products such as citric acid or clove oil. When the concentration in the product is higher than 5%, handling might be hazardous, therefore precautions should be taken [16, 63, 69, 92]. A further natural product is corn gluten meal, a by-product from wet milling of *Zea mays* L. (Poaceae) grains. This meal is used as a fertiliser and pre-emergence herbicide with no activity on existing weeds; it inhibits germination and development from the seeds. The postulated mode of action is the production of five dipeptides and one pentapeptide by microbial hydrolysis of the gluten. These peptides are phytotoxic by affecting nuclear development, membrane integrity, and cell wall formation. Due to the required hydrolysis, the herbicide is a slow-release product. Many different brands are available on the market, as for example Concern Weed Prevention Plus™, WeedBan™, or Supressa™. The use of this product is often cost prohibited, since huge amounts are needed to have an effect (more than 2 tons per hectare) [16, 40, 63, 69, 92].

An important plant-derived herbicide was discovered in the Myrtaceae family. Many Myrtaceae plants contain β-triketone secondary metabolites such as leptospermone (**45**)

(Figure 17), in particular *Leptospermum scoparium* J. R. Forst & G. Forst., commonly called manuka, and the bottle brush *Callistemon citrinus* (Curtis) Skeels. Some scientists observed that the amount of grass growing under *C. citrinus* was highly reduced. Subsequent investigation revealed that leptospermone, mainly contained in the essential oil, was responsible for the pre- and post-emergence herbicidal activity [42]. The compound bleaches the foliage through targeting of the *p*-hydroxyphenylpyruvate dioxygenase (HPPD). HPPD is essential for the synthesis of plastoquinone and tocopherols in plants. Plastoquinone is involved in the carotenoid synthesis and the lack of these pigments leads to bleaching of chlorophyll. The pre-emergence activity is explained by the persistence of the compound in soils. When pure leptospermone is applied, the DT_{50} is 15.1 ± 0.5 days while when it is applied as manuka essential oil the dissipation time is slightly longer (DT_{50} = 18.2 ± 0.6 d) due to matrix effects. Leptospermone also shows activity against some bacteria, such as *Clostridium* spp. and is considered as safe for the environment. These observations led to the development of systemic synthetic derivatives, such as sulcotrione (**46**) (Figure 17) or mesotrione (Callisto™ or Tenacity™) [16, 36, 40, 42, 51, 53, 59, 60, 63, 119-123]. Another compound present in many plant essential oils, 1,4-cineole, possesses phytotoxic properties. A synthetic derivative, racemic cinmethylin (**47** and **48**) (Figure 17), was then developed as a herbicide (Argold™) presenting a good ecotoxicological profile. The supposed mechanism of action is the inhibition of tyrosine aminotransferase enzymes, affecting finally the production of carotenoids [40, 119, 123-126].

Bialaphos (or bilanafos) (**49**) (Figure 17), a fermentation product of *Streptomyces hygroscopicus* (Jensen) Waksman & Henrici and *Streptomyces viridochromogenes* (Krainsky) Waksman & Henrici, was found to be a total herbicide applied post-emergence. The compound shows no *in vitro* activity, for the reason that it is metabolised by plants into *L*-phosphonitricine (ammonium glufosinate). This metabolite exerts a systemic (xylem and phloem) herbicidal activity and acts as an irreversible inhibitor of glutamine synthetase, inducing a reduced amount of glutamine and an increased amount of ammonia in the tissues. This blocks photosynthesis which results ultimately in plant death. No resistance has emerged so far. Bialaphos shows a moderate level of toxicity (acute oral LD_{50} to rats between 268 and 404 mg/kg) and is rapidly degraded by microbes, so that this product is considered to have a low environmental impact. The compound is commercialised under the name Herbiace™ and synthetic glufosinate is also available on the market as a racemic mixture of *L*- and *D*-

phosphinotricine (Liberty™, Basta™, or Ignite™), the *D*-enantiomer being inactive [16, 36, 37, 40, 42, 45, 59, 60, 69, 119, 123].

Finally, the selective contact herbicide endothall (**50**) (Figure 17) is an analogue of cantharidin (**51**) (Figure 17), a strong vesicant produced by the blister beetles, especially Spanish fly (*Lytta vesicatoria* L.). It acts as a strong protein phosphatase inhibitor. Commercial products include Aquathol™ (dipotassium salt) or Niagrathol™ (disodium salt). Endothall is moderately toxic to mammals and deleterious to some species of fish, but it is rapidly degraded in the environment [40, 119, 127-129].

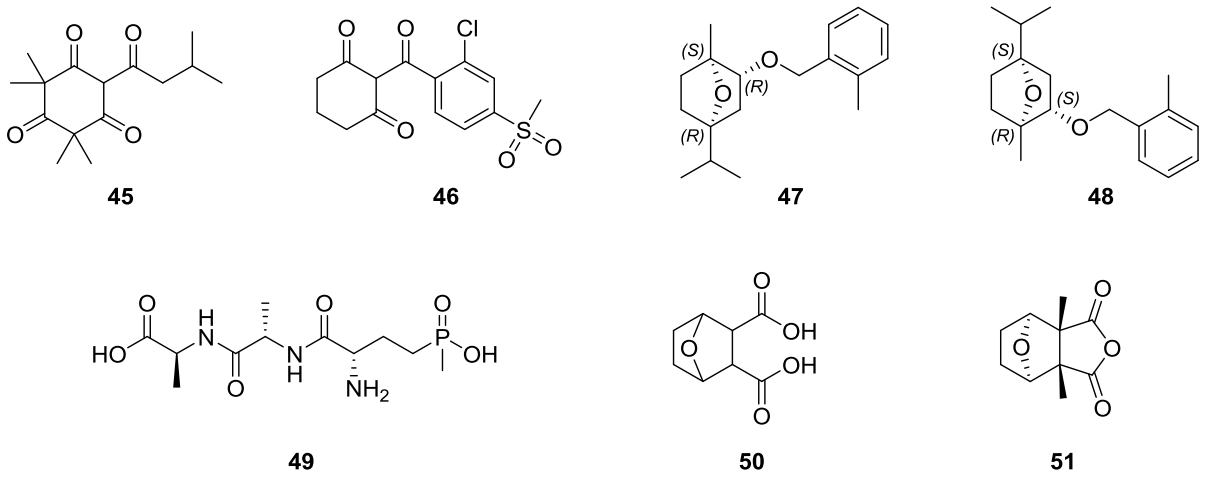

Figure 17: Leptospermone (**45**), sulcotrione (**46**), (1*S*,2*R*,4*R*)-cinmethylin (**47**), (1*R*,2*S*,4*S*)-cinmethylin (**48**), bialaphos (**49**), endothall (**50**), and cantharidin (**51**).

2.1.4. Plant oils and fatty acids with broad activity

Plant oils, essential oils, and fatty acids often exert unselective activity on several pest types. Sunflower, olive, canola, corn, or grape seed oils are highly effective against powdery mildew of apple trees (*Podosphaera lichotricha* (Ellis & Everth.) E. S. Salmon) and of grapevine (*Uncinula necator* (Schwein.) Burrill, anamorph *Oidium tuckeri*) [68]. In addition, plant oils are also potent contact insecticides/miticides to soft bodied organisms by physically disturbing their respiration and provoking alterations of the cell membranes. Another insecticidal effect is the hindering of feeding, since the plant is covered by an oily layer. An example of commercially available plant oil is Telmion™ containing rapeseed oil. Nevertheless, oils present some drawbacks such as causing some acute or chronic phytotoxicity and deleterious effects to beneficial insects and predatory mites [4, 12, 46, 63, 68, 69, 89, 130].

However, some oils have a better ecotoxicological profile. One example is fennel (*Foeniculum vulgare* Mill., Apiaceae) oil registered as a plant protection product, representing a good alternative to prevent powdery mildews [68]. Furthermore, a mixture of fish and sesame (*Sesamum indicum* L., Pedaliaceae) oils is used as a fungicide, miticide, and insecticide (Organocide™). By contact, it destroys the pathogen cell wall/membrane, and interferes with the attachment of the fungi to the plant [58]. Moreover, the oil extracted from Jojoba seeds (*Simmondsia californica* Nutt. and *Simmondsia chinensis* Link., Simmondsiaceae) is commercialised as an insecticide against whiteflies (Detur™) and as an antifungal agent against powdery mildews (E-Rase™). On whiteflies, the oil makes the eggs and immature life stages suffocate. Moreover, it is repellent against adults. The antifungal effect is exerted by blocking the access to oxygen of spores and mycelium [14, 16, 31, 69, 70]. Another product, Tecnobiol™, is a mixture of fatty acids (mainly linoleic and oleic acids) that is principally applied preventatively against grapevine downy mildew [4, 131].

In addition, several fatty acid salts (mainly sodium or potassium), commonly called soaps, are non-selective and non-residual total herbicides, moss and liverwort exterminators, insecticides, and fungicides. A broad range of products are available on the market, such as Naturell WK Herbicide™, Naturell WK Mosskiller™, Savona™, Neo-Fat™, M-Pede™, or Hinder™. Their composition is rarely extensively described, although the main constituent is generally oleic acid. The disruption of cell membranes is responsible for the activity. The soaps are harmful to soft-bodied predators, but their impact is generally minimal on honey bees, parasitic wasps, and ladybird beetle larvae. Furthermore, they are rapidly biodegraded, show low toxicity to mammals, and are safe for humans [16, 46, 63, 69, 95]. Another example of soap use is coconut soap (*Cocos nucifera* L. oil saponified, Arecaceae) against apple sooty blotch fungus. Its efficacy is lower than copper and insufficient in the case of high disease pressure, however its utilisation is recommended in organic apple production [68]. Coconut soap is also used to eliminate snails and slugs [92]. A specific fatty acid, pelargonic acid (nonanoic acid), is utilised as a non-residual, non-systemic contact herbicide with a broad spectrum, also controlling moss and liverworts. Commercial products are Scythe™, Racer™, or Slasher™ for example. The activity is expressed by cell membrane disruption and light-enhanced peroxidation of membrane lipids leading to rapid desiccation. Pelargonic acid is in particular found in the plant family Geraniaceae. The compound is considered as a herbicide with low environmental impact and toxicity [16, 40, 63, 69, 119].

Besides these, some essential oils from plants obtained by steam distillation possess a wide range of pesticidal activities, including herbicidal, insecticidal, miticidal, nematicidal, and fungicidal properties with low toxicity to mammals [12, 41, 46, 69, 132]. However, the problem with essential oils is that they volatilise rapidly, so that they are active only for a short time [16]. A few examples of oils used commercially are cited in the next paragraph.

Thyme (*Thymus vulgaris* L., Lamiaceae) essential oil, the active ingredient of Promax™, is used as preventive/curative soil fungicide and nematicide. Another product containing thyme oil (ThymeGuard™) is recommended as contact/systemic bactericide, fungicide, virucide, and insecticide [14, 58]. The essential oil of *Thymbra spicata* L. (Lamiaceae), the Mediterranean thyme, is commercialised under the name BioZell 2000B™ and is applied as a plant strengthener against bacterial and fungal diseases [4]. BacStop™ is a contact fungicide and bactericide containing a mixture of oils: clove (*Syzygium aromaticum* (L.) Merrill & Perry, Myrtaceae), thyme (*T. vulgaris*), cinnamon (*Cinnamomum* spp. Schaeff., Lauraceae), garlic (*Allium sativum* L., Amaryllidaceae), and peppermint (*Mentha* × *piperita* L., Lamiaceae) [14, 58]. Another mixture on the market is Sporan™, a broad spectrum fungicide containing rosemary (*Rosmarinus officinalis* L., Lamiaceae), clove (*S. aromaticum*), thyme (*T. vulgaris*), and peppermint oils (*M. piperita*) [14, 46, 58]. In addition, clove oil is used as a herbicide against poison ivy (*Toxicodendron radicans* (L.) Kuntze, Anacardiaceae) at a concentration of 12% in the product Poison Ivy Defoliant™. Clove oil is a non-selective foliar herbicide, which causes quick cellular membrane damages by contact. It does not show systemic properties and therefore only controls the above ground parts. Another commercial product, Matran™, is also sold as a herbicide. Finally clove oil, containing mainly eugenol, is applied to plant foliage as a deterrent to a wide range of insects in fruit and vegetable crops. Eugenol possesses irritant properties so that these products should be used carefully [14, 16, 35, 46, 69].

2.1.5. Current developments

Further efforts to discover and develop new pesticides based on natural products are ongoing. Some compounds are currently in late development. For instance, the fungicide fenpicoxamid (**52**) (Figure 18) is under investigation by Dow AgroSciences in collaboration with Meiji Seika Pharma to be applied principally on cereals. It is a synthetic derivative of UK-2A (**53**) (Figure 18), a compound isolated from fermentation broths of *Streptomyces* species. Fenpicoxamid and UK-2A both inhibit the mitochondrial respiration of the fungi by

binding to the Q_i ubiquinone site of the complex III instead of the Q_o site which is targeted by the strobilurins. The product, called Inatreq™ Active, should be accepted by the authorities in 2018 and available commercially in 2019-2020. Its toxicity is very low against non-target organisms, but it shows significant deleterious effects on some aquatic organisms [42, 133].

A potent broad-spectrum post-emergence herbicide, cornexistin (**54**) (Figure 18), is thought to be under development at BASF. The compound was isolated in Alberta (Canada) from the fungus *Paecilomyces variotii* Biourge & Bainier growing on elk excrements and acts by inhibiting the transketolase [42, 123, 134]. Another compound of interest as an herbicide could be the sarmentine (**55**) (Figure 18), isolated from *Piper* species (Piperaceae). This amide desiccates rapidly the foliage by inducing the loss of plasma membrane integrity by several mechanisms of action [135].

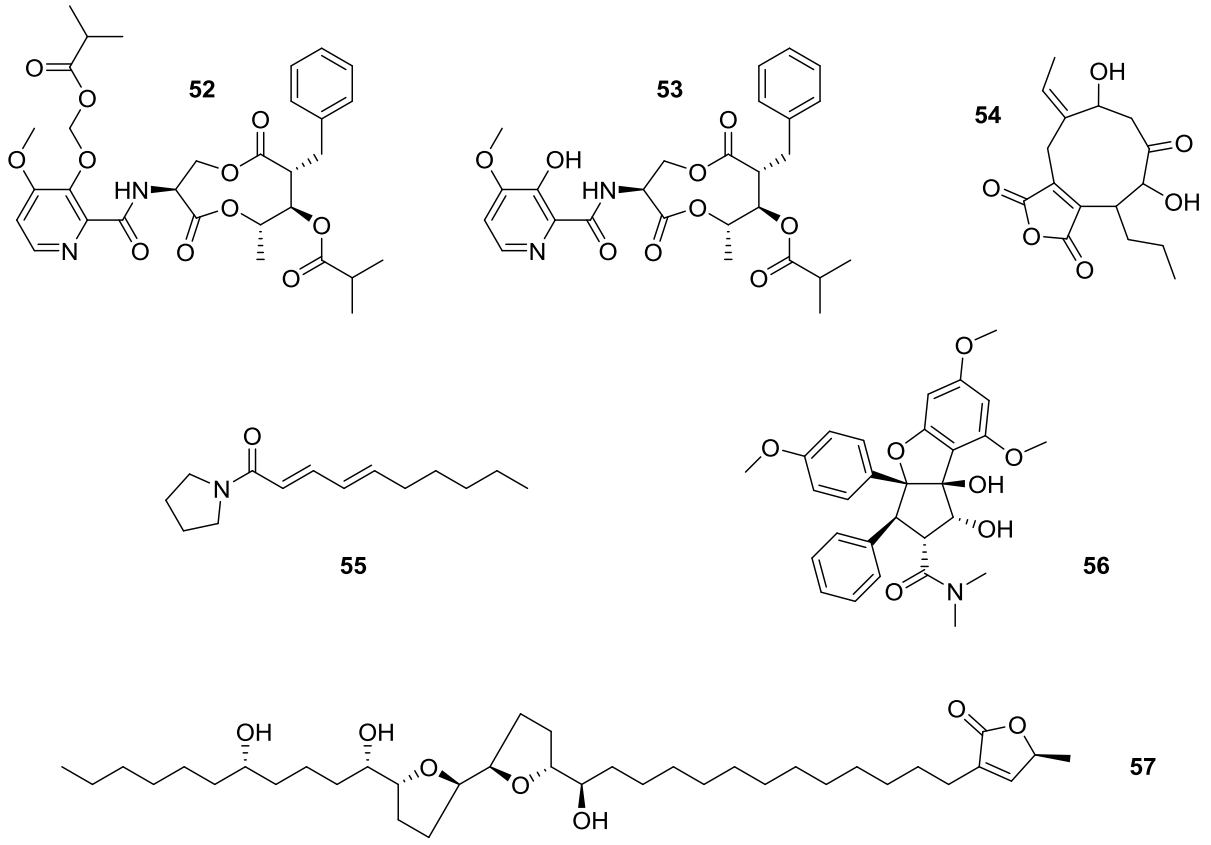

Figure 18: Fenpicoxamid (**52**), UK-2A (53), cornexistin (**54**), sarmentine (**55**), rocaglamide (**56**), and squamocin (**57**).

Furthermore, some interesting insecticidal natural products are rocaglamides and acetogenins. Rocaglamides (**56**) (Figure 18) which are found in the genus *Aglaia* (Meliaceae) inhibit protein synthesis, and are slightly more potent than azadirachtin against some species

[46, 51, 97]. Acetogenins (e.g. squamocin (**57**) (Figure 18)), isolated from the Annonaceae family, block the energy production in the mitochondria by inhibiting the NADH-ubiquinone reductase (complex I) [35, 46, 49, 97, 136].

2.2. Organic farming

Organic farming is a holistic production system that encourages the health of agro-ecosystem [137]. The International Federation of Organic Agriculture Movements (IFOAM), worldwide organisation, defines organic agriculture as follows:

> "Organic agriculture is a production system that sustains the health of soils, ecosystems and people. It relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects. Organic agriculture combines tradition, innovation, and science to benefit the shared environment and promote fair relationships and a good quality of life for all involved."
>
> Copyright IFOAM - Organics International, Charles-de-Gaulle-Str.5, 53113 Bonn, Germany, www.ifoam.bio [138]

According to IFOAM [139], four principles drive organic farming development, namely health, ecology, fairness, and care:

- Health: maintenance and improvement of soil, plant, animal, human, and planet health, which are considered as one and indivisible. Taking this into account, pesticides, fertilizers, animal drugs, and food additives with potential deleterious effects should be avoided.
- Ecology: preserving and enhancing environmental quality by respecting the natural life cycle and ecological systems. Biodiversity is promoted, and, for example, GMOs are strongly prohibited. Also, resources ought to be protected by recycling and competent management of materials and energy.
- Fairness: equity, respect, justice, and stewardship of the shared world. Fairness should be guaranteed at all levels and to all parties involved, with a good quality of life. Organic agriculture should also play a role for food autonomy and

diminution of poverty. Additionally, the physiology, natural behaviour, and well-being of animals must be respected. This principle of fairness should be applied to natural resources that have to be used in the appropriate social and ecological manner.

- Care: precaution and responsibility in managing organic agriculture to be able to protect the environment, and the well-being of current and future generations.

The emergence of organic farming was driven by the wish of the consumer for safe and healthy food produced in a way to guarantee sustainability [140-142]. Pioneers, such as Rudolf Steiner, developed the organic concepts during the inter-war period, when advanced farming faced a crisis [143, 144]. A tremendous increase of organic research and farms occurred during the second part of the 20th century. At the beginning of its development, organic production was condemned by a part of the scientific community. For instance, some members of the American Association for the Advancement of Science (AAAS) strongly criticised organic farming during their annual meeting in 1974 [145]. According to the Washington Post on the 28th of February 1974 [146], organic farming was described by the AAAS as 'scientific nonsense' being the domain of 'food faddists and eccentrics'. AAAS scientists also declared that 'organic myth was counterproductive to human welfare, because the myth leads to a rejection of procedures that are needed for the production of nutritious food at maximum efficiency', and were 'eroding gains of decades of farming advancements'. Nevertheless, a few years later, a seminal research paper was published by *Science* (AAAS Journal) showing that organic farms are proficient and economically competitive in comparison with conventional farms [147]. In 2015, 1.1% (50.9 million hectares) of the world's agricultural land was under organic cultivation, with 22.8 million hectares in Oceania and 17.7 million in Europe. On the other hand, there are almost 2.4 million of organic producers and Asia represents 35% of them with the biggest proportion in India (585'000 farmers), followed by Africa 30% (mainly in Ethiopia, 203'602), and Latin America 19% (mainly in Mexico, 200'039) [148].

Reganold and Wachter [149] reviewed 55 studies, and qualitatively compared the performance of organic and conventional farming with a "flower petal" chart graphic (Figure 19). The review demonstrated that organic farming has a better impact in terms of sustainability. Even if the yields are lower, the maintenance of fertility and biodiversity makes the whole process globally more efficient [139, 142, 149-152]. With the tremendous increase in yield (47% between 1985 and 2005) [11], conventional farming has a negative

impact on the arable surface, one third of which has already been lost due to erosion [149, 153, 154]. This leads to considerable increase of fertilizer use to palliate the loss of fertility [30]. A severe impact on biodiversity is also observed [21, 33, 149, 152, 155]. Furthermore, huge health and environmental external costs are generated [21, 32, 33].

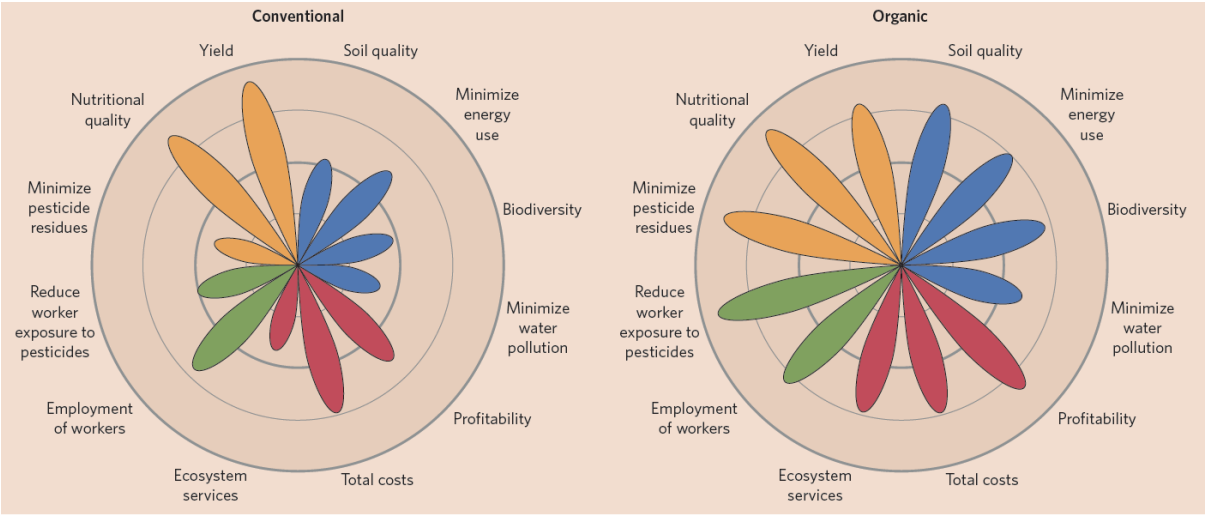

Figure 19: Organic farming compared to conventional farming in the four main spheres of sustainability. The four spheres are represented with: yellow petals corresponding to production, blue petals to environmental sustainability, red petals to economic sustainability, and green petals to well-being. The lengths of the twelve petals indicate the level of performance relative to the four circles representing 25, 50, 75, and 100%.

In addition, it has been proven in a review written by Lairon in 2010 [156] that organic food is of better quality. The main points highlighted are: (i) there is no pesticides residues in 94-100% of organic products, (ii) the mycotoxins contamination of organic cereals is similar to cereals produced conventionally, (iii) the nitrates content of vegetables is approximately 50% lower, (iiii) the quantity of dry matter, minerals (such as Fe or Mg), and antioxidant is higher in organic plant products, and (iiiii) the animal products have more polyunsaturated fatty acids.

Nevertheless, some scientific communities are still sceptical about food security and sustainability with the development of organic agriculture [13, 150, 157-160]. The main argument is that the obtained yields represent around 80 % of conventional production yields,

due to the prohibition of soluble mineral fertilizers and synthetic pesticides [158, 161]. More land is therefore needed to reach the same production level, which means that the surface of natural ecosystems will drop further, even if the biodiversity would be increased on and around the agricultural land [158]. Another aspect is the competition for the restricted areas which have sufficient amounts of organic nutrients, and for limited organic manure [152, 157]. To enrich soil nitrogen, which is one of the most important nutrients, organic manure is spread or legume intercrops are cultivated [152]. The first solution is limited, as aforementioned, by the amount produced by cattle, and the second one is problematic because of the disruption of the production [157, 158]. There is a high need for research, development, and technology transfer to overcome these issues [140, 142, 149, 151, 152, 162].

2.2.1. The use of copper as pesticide

Copper salts possess an exceptional broad spectrum of activity against many organisms (bacteria, algae, fungi, molluscs, mites, and viruses). The first recorded use in plant protection was in 1761 with the finding that soaking seeds in copper sulphate solution prevented seed-borne fungi. However, the breakthrough was at the end of the 19th Century with the discovery of Pierre-Marie-Alexis Millardet in 1882. The scientist detected the inhibitory activity of copper sulphate mixed with lime and water against grapevine downy (described in Chapter *Plasmopara viticola*). This finding was a case of serendipity since the first intention of Millardet for spraying this mixture was to dissuade people passing by of eating the grapes close to the paths, since it has a visible blue colour and a bad flavour. Then, he observed that the sprayed vines were exempt from downy mildew, whereas the rest of the yard was infested. This preparation has then been called Bordeaux mixture and has the advantages to stick to the leaves and to be coloured; this allows the easy verification of the product repartition after spraying [68, 83, 95, 163].

Copper salts dissolved in water release copper ions (Cu^{2+}) which permeabilise the cell membrane or wall leading to a leakage of cellular solutes and impaired viability. Once the ions enter the cytoplasm, they have the ability to bind to DNA and disorder the helical structure. In addition, copper denaturises proteins and hinders their biological activity leading to the dysfunction of several enzymes. The different modes of actions are illustrated on Figure 20 [6, 68, 163]. To permit a longer lasting activity on the plants, less soluble salts have been developed to release the ions progressively [68]. If the applied dose is too high or if the

plant surface dries too slowly, copper shows phytotoxicity; the fruits russet and the leaves become necrotic [68]. Despite its long and abundant utilisation, copper is still effective on main crops. Only a reduction of activity on bacterial diseases (e. g. tomato) has been observed after long-term applications [68].

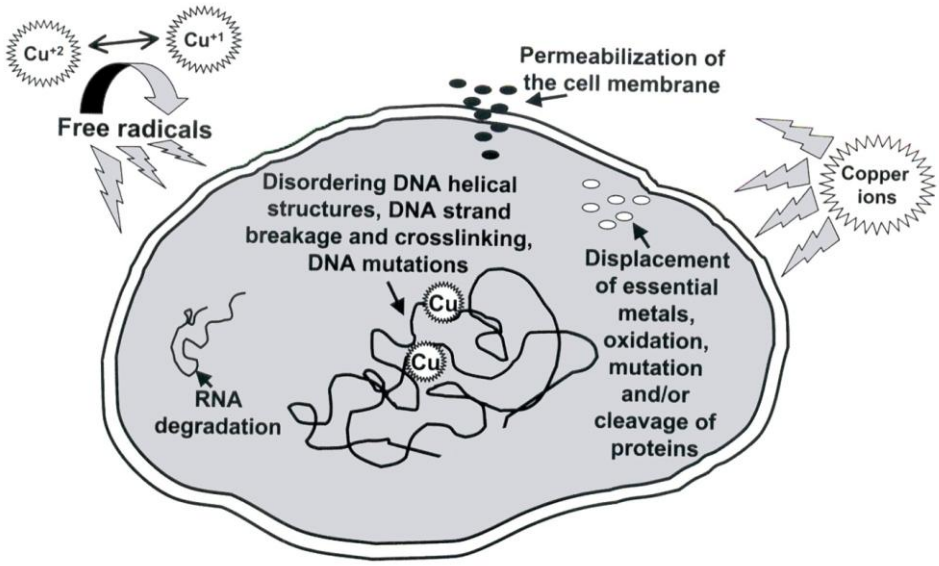

Figure 20: Copper mechanisms of toxicity on microorganisms.

Reproduced from [163]

For humans, animals, and plants, copper is a fundamental micronutrient required for a broad range of metabolic processes [164, 165]. However, the applied amounts in agriculture exceed the natural uptake so that copper accumulates in soils with a negative environmental impact [6, 61, 68, 83, 95, 165, 166]. In orchards, it was shown that the ecology of the soil is perturbed and the earthworm populations are decreased [167-171]. Nevertheless, copper salts can be considered safe to humans in the quantities used as pesticide [6, 163].

Copper is still allowed in organic farming, due to the lack of efficient alternatives against oomycetes, ascomycetes, and bacteria [166, 172-174]. The quantities pro hectare authorised are drastically reduced in comparison with traditional agriculture [4, 5]. Even if it was possible to decrease the amounts applied thanks to the improvement of formulation, application techniques, and timing of sprayings [3], finding substitutes to copper remains necessary.

2.3. Identification and characterisation of natural products with antifungal activity against plant pathogens

Discovery of bioactive natural products is a tedious approach involving many steps. The procedure to find extracts or compounds active against plant pathogens is illustrated on Figure 21.

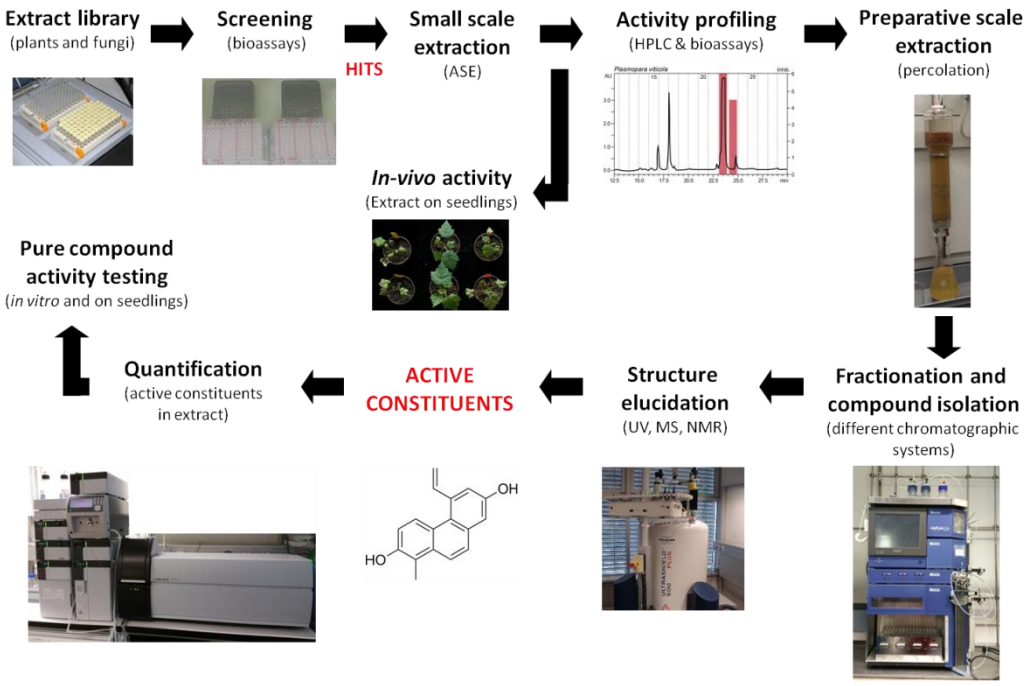

Figure 21: Approach to identify and characterise bioactive natural products.
Pictures: J. Ramseyer, University of Basel, and FiBL

At first, an *in vitro* screening of the library is performed, as described in Chapter 2.3.1. Thereafter a small scale extraction of the active plants (mg amounts of extract) follows to perform activity profiling (Chapter 2.3.2) and testing *in vivo* on seedlings (Chapter 2.3.3). If the previous steps are successful, a preparative scale extraction is carried out to gain grams of the extract. Bioassay-guided fractionation and isolation are performed on this extract (Chapter 2.3.2). Once the substances are purified by different chromatographic procedures [175, 176], they are identified and characterised with the help of various spectroscopic techniques, such as ultraviolet (UV), high-resolution mass spectrometry (HR-MS), microprobe nuclear magnetic resonance (NMR), electronic circular dichroism (ECD), and/or single-crystal X-ray diffraction [177-181]. Afterwards, the pure compounds are tested *in vitro*

with the same protocol as for the library screening (Chapter 2.3.1) and *in vivo* on seedlings in semi-controlled conditions if the amount isolated is sufficient (Chapter 2.3.3). Quantification of the active compounds in the extract can be performed to determine the concentration of the extract formulation used to spray the plants. If the extract and the pure compounds are deemed sufficient potential for large scale application, they are then tested in field conditions (Chapter 2.3.4).

2.3.1. Library-based screening against selected plant pathogens

Pesticides of botanical origin could represent an important tool to reduce the burden of agrochemicals [12]. To discover new active natural compounds, the first step is a screening of different extracts. For this purpose, an in-house library of more than 3000 extracts from approximately 800 plants and 100 fungi was screened *in vitro* against plant pathogens. The utilisation of a library permits a straightforward process to discover new directions.

To generate the library, a few grams of powdered plant material (such as leaves, bark, roots, or fruits) are extracted by Accelerated Solvent Extraction (ASE). Each sample is extracted sequentially with three solvents of increasing polarity to allow a pre-fractionation of the constituents (lipophilic, intermediate, and polar). In general, the solvents are petroleum ether (or dichloromethane), followed by ethyl acetate, and finally methanol. The extracts are then stored as 10 mg/mL DMSO stock solutions in 96-well format racked microtubes at -80°C. Each microtube possesses a barcode to allow a sure identification, and each plate has three identical replicates as back-ups. Daughter plates are generated for testing. The preparation of library and daughter plates is supported by a robotic system. A detailed description of the library generation was published by Potterat and Hamburger [7].

The *in vitro* screening of extracts, illustrated on Figure 22, was performed in 96-well plates at three concentrations (490, 49, and 4.9 µg/mL) against three major agricultural pathogens: *Plasmopara viticola*, *Venturia inaequalis*, and *Phytophthora infestans*. The pathogen characteristics are described below. After incubation, scoring is done manually for each well under a binocular magnifying glass, and activities of the three concentrations are summed up. A complete description of these assays can be found in Chapter 3.1 (2.2 Bioassays).

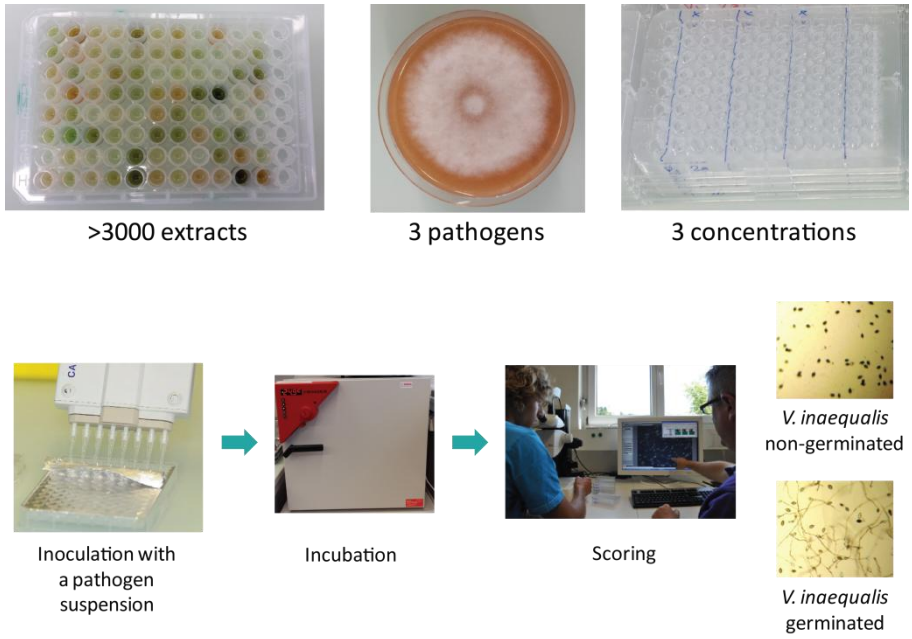

Figure 22: *In vitro* screening of the library.

Pictures: FiBL and J. Ramseyer

Plasmopara viticola

Plasmopara viticola (Berk. & M. A. Curtis) Berl. & De Toni is endemic in North America and causes grapevine downy mildew. It was first recorded in Europe in 1878. Its introduction probably happened after the epidemic of phylloxera (mentioned in Chapter 2.1.2). To palliate this destruction, France imported American grape rootstocks resistant to the insect to replant their vineyards. In the first half of the 20th century, *P. viticola* ravaged the vines in Central Europe, predominantly in France, Germany, and Switzerland. In 1915, for instance, 70% of the grape production was destroyed. In North America, the grapevine species are more or less tolerant to downy mildew. However, in Europe, since *Vitis vinifera* L. evolved without it, its susceptibility is exceptionally high [3, 54, 83].

P. viticola is an oomycete (peronosporales order) and obligate parasite. Its life cycle is illustrated on Figure 23. The sexual oospores are produced during summer or early autumn (1, 1'), overwinter in leaf fragments on the soil or embedded in the ground (2), and in spring they generate a sporangium for primary infection (3). Rain splash and wind spread these sporangia on the leaves, and then the asexual zoospores are released (4). The zoospores possess two flagella and swim on the wet leaf surface after rain or during morning dew to reach the stomata. Zoospores and sporangia are really sensitive to desiccation. Exposed to

sunlight and dryness, they die in 2 to 3 hours, but in appropriate conditions, could survive more than 24 hours on the leaf surface. Afterwards, the zoospore encysts close to the stoma and creates a germ tube that enters the plant tissue through the stoma (5). This indicates that all green tissues with active stomata can be affected, including young stems, leaves, inflorescences, and young berries. Thereafter, globose haustoria and mycelium are produced into the tissue (6). The haustoria permit the absorbance of nutrients from the host tissue. Finally, new sporangia are formed on the underside of the leaf and on the berries (7) after 6 to 14 days of incubation depending on weather conditions (minimum 98% of relative humidity and minimal temperature of 13°C). Seven hours are needed to complete the sporulation process that starts in the darkness. These sporangia permit secondary infections (8) [3, 54, 182].

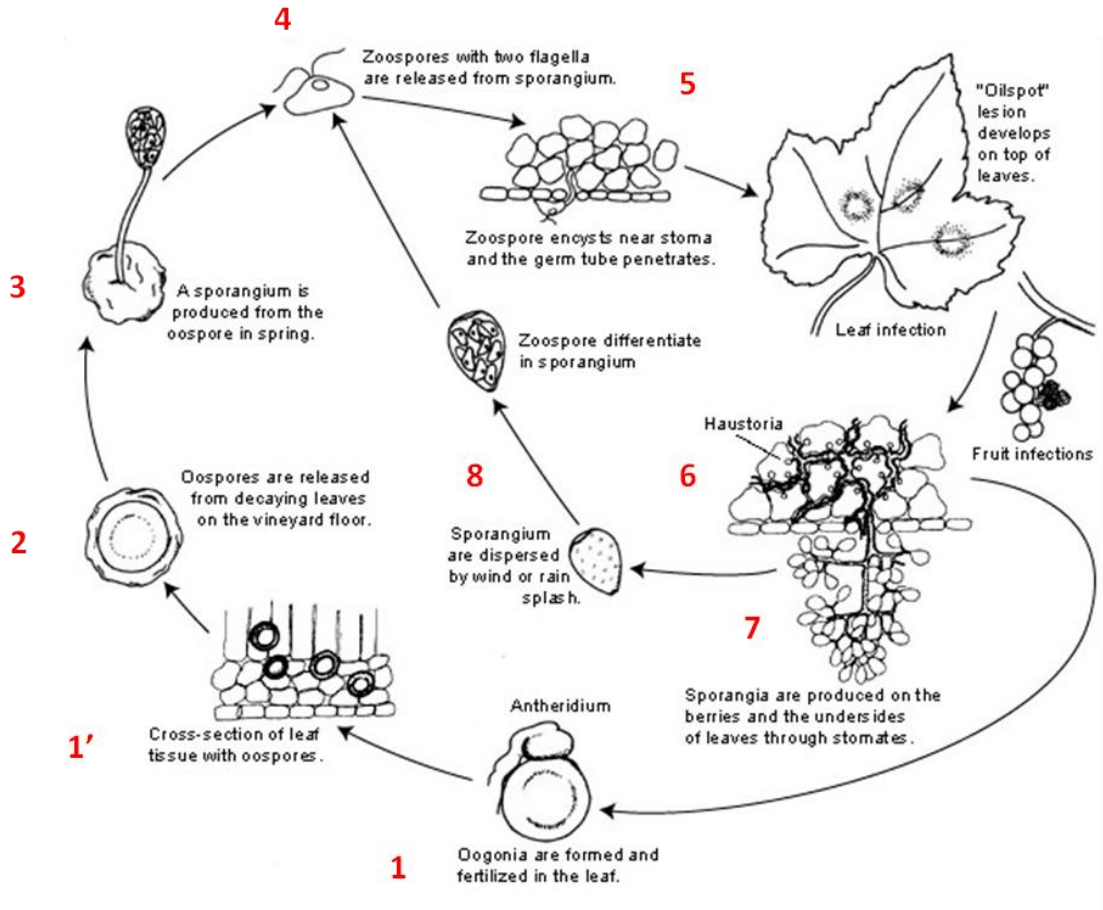

Figure 23: *Plasmopara viticola* life cycle.

Adapted from [182], www.apsnet.org

The first symptoms, appearing five to seven days after infection, are characterised by small, pale yellow, and irregular “oil spots” on the upper side of the leaf (Figure 24, picture 1). This is followed by sporangia production that appears like downy whitish mould on the underside (Figure 24, picture 2); the vernacular name downy mildew originates from this symptom. Afterwards the lesions become necrotic and the sporophores turn dark grey. These lesions habitually coalesce, create large dead areas, and often implicate defoliation. The inflorescences are highly sensitive, and the infected berries are covered with downy growth (Figure 24, picture 3), turn purple, and shrink. Frequently the entire bunch is ruined (Figure 24, picture 4). The infected stems become distorted or thickened because the cells enlarge and a large amount of mycelium is present in the intercellular space. After this, the sick cells die, collapse, and create concave, brown spots on the stems. Stems, leaves, and fruits get less susceptible with ageing [3, 54, 182].

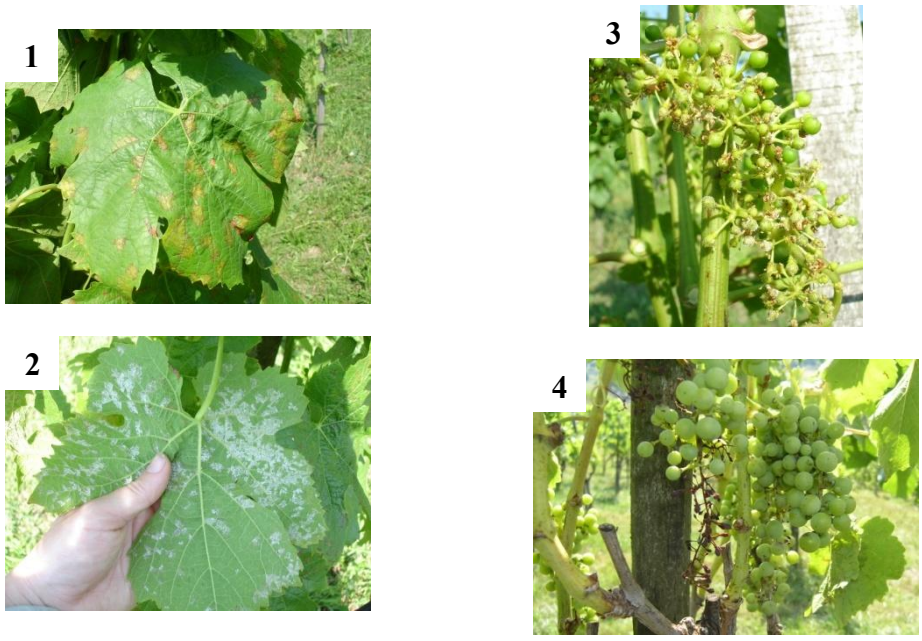

Figure 24: Symptoms of *Plasmopara viticola* on leaves (1, 2) and fruit bunches (3, 4). Pictures: FiBL

Once the epidemic begins, it extends extremely quickly and can cause complete defoliation and berry loss, if nothing is appropriately done to stop it. The severity of the damages is also influenced by the length of cool and wet weather periods that allows prolific sporulation. Some agricultural practices can reduce the incidence. Firstly, vines should be planted on well-drained soils and in regions with good air movement. Secondly, the stocks

should be planted in rows, to allow the natural flow of the air. These measures permit faster drying of the leaves. Other points are to try to maximally reduce the leaf litter and to prune the stocks to decrease the quantity of overwintering inoculum. Furthermore, since several North American grapevine species are resistant to *P. viticola*, interspecific hybrids of *V. vinifera* with resistant species show good results. However, consumers are reluctant about these unknown hybrids, so that a chemical control of downy mildew is unavoidable. In traditional agriculture, *P. viticola* is controlled by various systemic fungicides (such as metalaxyl, mancozeb, or strobilurins). In organic agriculture, the disease control is performed mainly with copper products, since barely any alternatives have been authorised [3, 54, 182].

Venturia inaequalis

Venturia inaequalis (Cooke) G. Winter is an ascomycete causing apple scab on *Malus domestica* Borkh. species. The disease is spread worldwide, but more critical in regions with cool, humid spring and summers. It is the most important disease of apples. Around 100 cultivars are genetically resistant to this scab, but the most popular are moderately to highly vulnerable [54, 183].

The pathogen life cycle is described on Figure 25. *V. inaequalis* overwinters in fallen fruits or leaves on the orchard ground (1). Pseudothecia, sexual fruiting containing 50 to 100 asci each, are produced during winter and early spring, when weather is moist and between 5 to 15°C (2). The mature ascospores are then released from the asci during rainy days, and trigger the primary infection on young terminal leaves and fruits by pricking the cuticule (3). Afterwards, the asexual conidia are produced on the surface of infected tissues (4). These conidia are then diffused by wind and rain creating secondary infection on further plant parts (5) [183].

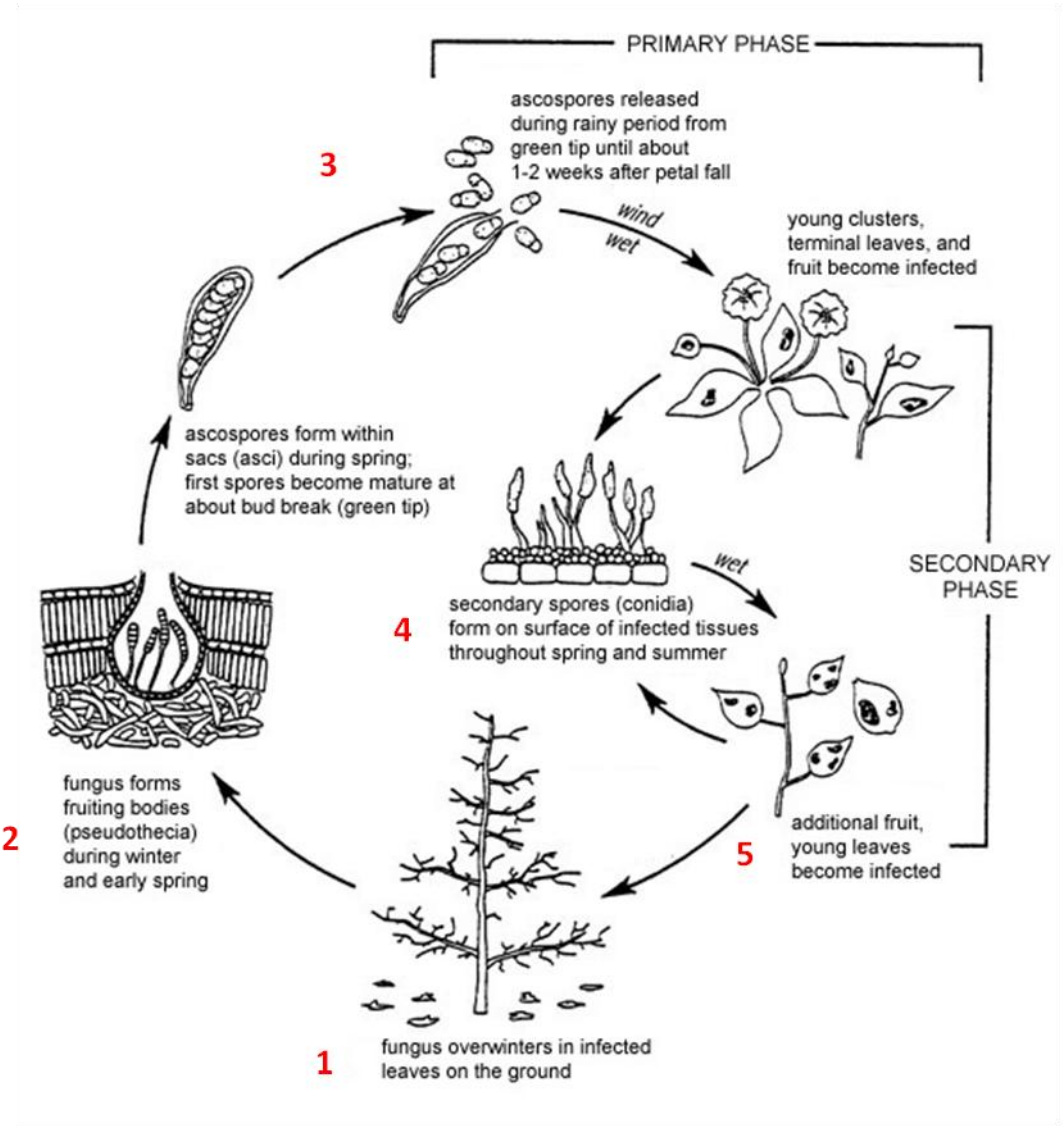

Figure 25: *Venturia inaequalis* life cycle.
Adapted from [184], www.apsnet.org

Apple scab affects the leaves and fruits. The first symptoms are light, irregular, olive-coloured spots on the young leaves. The lesions then turn rapidly olive green and get velvety. In the next stage, they show a metallic black colour and sometimes a little elevated (Figure 26, picture 1). It can lead to defoliation. The effects on the fruits are deformation, diminution of size, premature drop, and reduced storability. They show similar lesions as the leaves, but the dark spots are blistered, scabby, and cracked (Figure 26, picture 2). If no appropriate control is applied, the total fruit value is decreased by 70% or more, no marketable fruits can be harvested, and the next season's productivity could be impacted. After two to three years

of consecutive infection, the tree gets weakened and therefore more sensitive to frost, insects, or other diseases [54, 183, 184].

Figure 26: Symptoms of *Venturia inaequalis* on a leaf (1) and a fruit (2).
Pictures: FiBL

In organic farming, apple scab is mostly managed with the aid of copper and sulphur fungicides which only have a preventive activity. In traditional agriculture, various fungicides including captan, mancozeb, fenarimol, myclobutanil, thiophanate-methyl, or benomyl are good at controlling *V. inaequalis*. Some of them are only preventive, and not curative [54, 183].

Phytophthora infestans

Phytophtora infestans (Mont.) de Bary is an oomycete causing potato and tomato late blight. The pathogen also affects some other Solanaceae, but the economical significance of these is lower. The disease is found in almost every part of the world where potatoes (*Solanum tuberosum* L.) and tomatoes (*Solanum lycopersicum* L.) are produced. With wet and cool weather conditions, it can blight and rot all the aboveground parts of a field in one or two weeks, and gives off a typical odour [54, 185, 186]. Etymologically, the name "*phytophthora*" come from the Greek words "*phyto*" and "*phthora*" meaning "plant" and "destroyer", respectively. In the 1840s, *P. infestans* triggered the famine in Ireland by destroying the potato crops during several consecutive rainy and cold years. Heinrich Anton de Bary, a German scientist, was the first to prove that late blight was due to a pathogen and not to spontaneous decomposition [186].

A simplified life cycle of *P. infestans* is shown on Figure 27. The pathogen produces sexual oospores able to stay alive for more than four years in the ground. This could happen only when the two mating types are present (1). These oospores then develop sporangia with appropriate weather conditions (2). In warm countries, primary infection by fruiting oospores is ordinary. In opposition, in regions with winter frost, the contamination mainly occurs by surviving mycelium in infected volunteer plants and seed tubers. In this case, the inoculum consists of asexually produced sporangia in young plants in spring (3). Sporangia can be carried by wind and rain splash on long distances during the night and when days are rainy and overcast (low UV radiation). The penetration in the leaves or the tubers by the pathogen can go through two pathways. On one side, when the temperature is between 12 to 15°C, the sporangium releases flagellated zoospores, and the zoospores encyst on the tissues, before producing appressoria-like bodies and hyphae (4). On the other side, when the temperature is above 15°C, the sporangium has the possibility to germinate and enter the plant by itself (5). Finally, new sporangia are produced from the infected leaves (6). To produce new spores, the pathogen necessitates 3 to 4 days on really susceptible cultivars under ideal conditions (18 to 23°C and wetness). During precipitation, the spores can be washed on the soil surface and reach tubers that are not well covered [54, 185, 186].

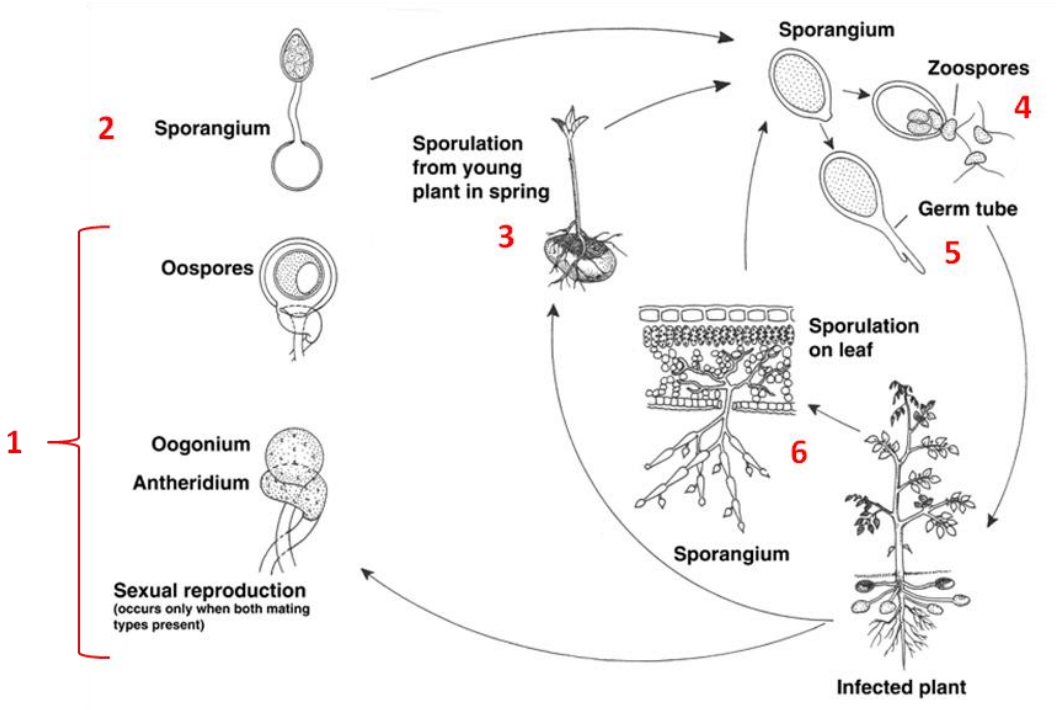

Figure 27: *Phytophthora infestans* simplified life cycle.

Adapted from [186], www.apsnet.org

Symptoms on potato plants appear on the foliage and are at first water soaked spots, enlarging rapidly in humid conditions, and turning to brown, blighted areas (Figure 28, pictures 1 and 3). A downy whitish sporulation on the borders of the lesions and on the underside of the leaf occurs (Figure 28, picture 1, pointed by the red arrowhead). The leaf then rapidly dies, and wilts. When the weather gets dry and warm, the pathogen is checked, but not killed; the lesions of the leaves do not enlarge further, but turn black, coil, and droop; no sporulation is observed. As soon as the rain comes back, the process restarts rapidly. On the stems, the infection begins on axles and lesions turn black (Figure 28, pictures 2 and 3). The tuber symptoms start with brownish or purplish patches that are constituted of dark flesh (Figure 28, Picture 4), and thereafter dries and hardens. If the infection destroys the foliage early in the plant development, a complete loss of yield can occur, because the tubers are not able to develop. On the other hand, when the infection is late, the field looks destroyed, but the yield is not too much affected since the tubers are almost ready to be collected. However, even if the tubers are healthy, they can be contaminated during harvest and rot during storage by secondary infection of soft rot bacteria [54, 185, 186].

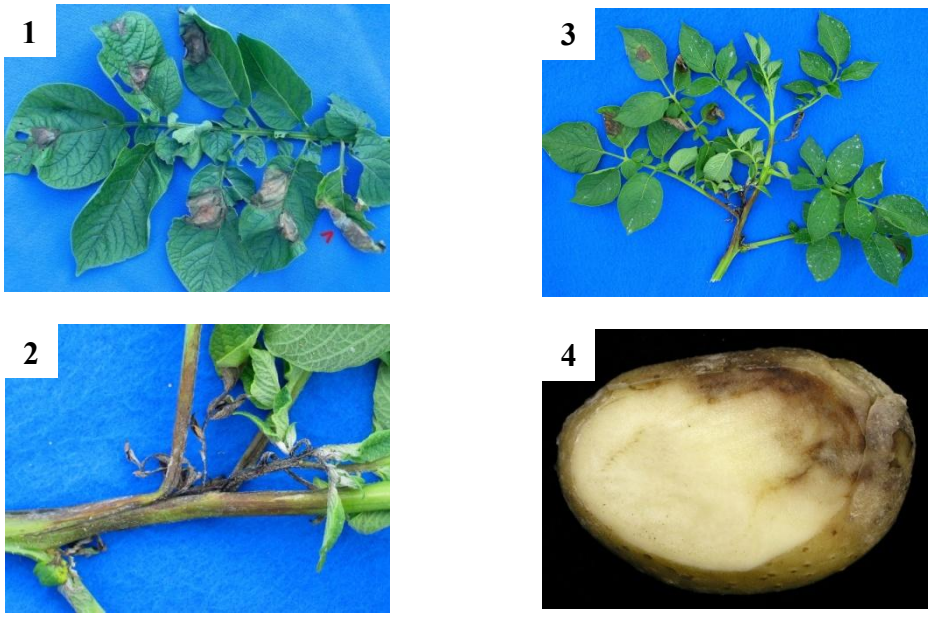

Figure 28: Symptoms of *Phytophthora infestans* on potato leaves (1, 3), stems (2, 3), and tubers (4).
Pictures: M. McGrath and S. Johnson

Symptoms on the leaves and stems of tomato plants are comparable as on potatoes (Figure 29, pictures 1 and 2, respectively). The infected tomato fruits show dark and hard

lesions (Figure 29, pictures 3 and 4). The tomato crop may also be quickly entirely destroyed, and the fruits are getting sensitive to soft rot bacteria as well [186].

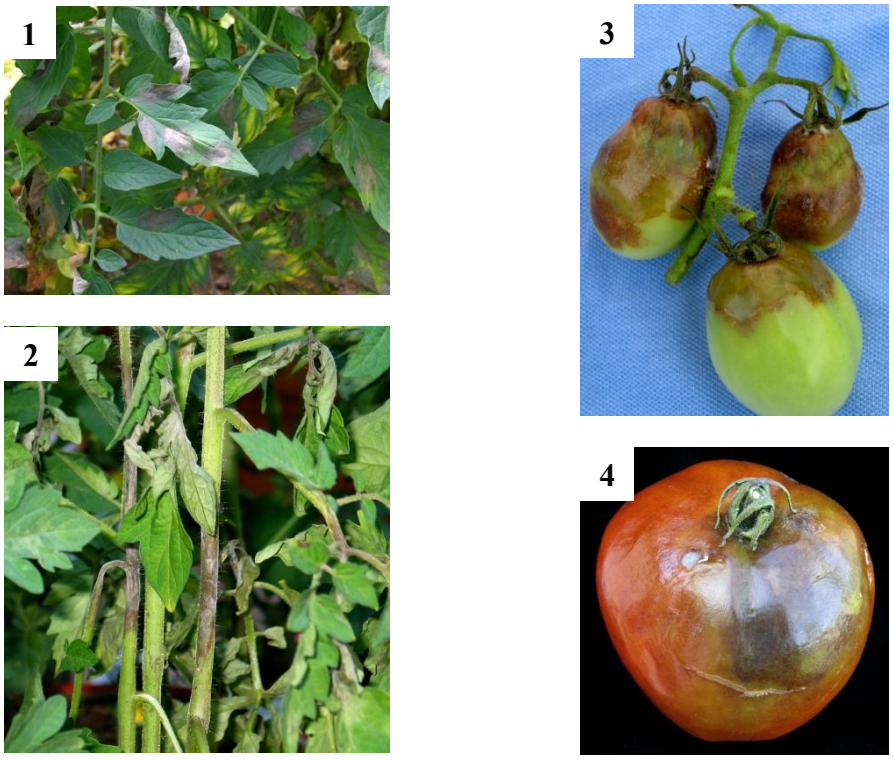

Figure 29: Symptoms of *Phytophthora infestans* on tomato leaves (1), stems (2), and fruits (3, 4).
Pictures: M. McGrath and S. Johnson

Resistant varieties, sanitary measures, and well scheduled fungicide sprays permit the management of *P. infestans* infection. For example, in potato cultivation, the tubers used as seeds should be disease free. The dumps and cull piles around the fields should be burned before planting time or buried deeply (more than 1 m), and volunteer plants should be destroyed as well to avoid any contamination. Furthermore, the area of cultivation should provide good air movement and good drainage to reduce the moisture as much as possible. In addition, crop rotation should be applied, and the survival of spores in the soil should be taken into account. Then, the most resistant cultivars should be cropped, but the ones preferred by the consumers are unfortunately sensitive to the disease. Before potato harvest, it is important to remove the aerial parts, to avoid an accidental contamination of the tubers by the infected leaves. The fungicides used in traditional agriculture include metalaxyl, mancozeb, chlorothalonil, and different copper formulations. In organic farming, the control is performed mainly by copper products [54, 185, 186].

2.3.2. Activity profiling and compounds identification

To select which active hits from the *in vitro* screening to further investigate, a literature research is performed. This helps to realise if an interesting plant already has activity against phytopathogens or toxicity issues reported. If this is the case, the corresponding extract is not further considered. Another important point is the availability of plant material, and the possibility to obtain large amounts in case of eventual commercialisation.

Once an extract is selected, it is submitted to a procedure referred to as HPLC-based activity profiling. The extract is fractionated time-based by analytical or semi-preparative HPLC. Usually, with analytical HPLC, 900 µg of crude extract are injected in three portions and fractions collected every 60 sec (typically at a flow rate of 0.4 mL/min with column dimensions of 3.5 µm, 150 × 3.0 mm i.d.). On the other hand, with semi-preparative HPLC, generally 20 mg are injected in two portions and fractions collected every 90 sec (usually at a flow rate of 4.0 mL/min with column dimensions of 5 µm, 150 × 10 mm i.d.). The total amount of sample injected is determined by the sensitivity of the assays. Afterwards, each obtained fraction is tested *in vitro* with a similar protocol as for the screening of the library (as described in Chapter 2.3.1). Then, the activity results are combined with the UV and MS chromatographic data to identify the peaks of interest. This procedure of HPLC-based activity profiling is widely described in different articles from Potterat and Hamburger [7, 187-189].

If the matrix is not too complex and the active peaks well defined, purification can be performed directly on the crude extract by preparative (typically at a flow rate of 20 mg/mL with column dimensions of 5 µm, 150 × 30 mm i.d.) or semi-preparative HPLC. In the opposite case, a preliminary fractionation by open column chromatography is necessary. If possible, the active peaks are localised in the generated fractions and purified by HPLC. In cases of highly complex mixtures, an *in vitro* testing of these fractions can be necessary, followed by the submission of the active fractions to activity profiling, prior to isolation.

2.3.3. Tests on seedlings

Once *in vitro* activity is confirmed and the active compounds identified, the extracts and pure compounds (obtained in sufficient amounts) undergo activity testing on seedlings of grapevine, apple tree, and/or tomato. The extracts are formulated to enhance their solubility. The reference antifungal product for comparison is copper hydroxide.

First, the seedlings are grown in a greenhouse until they reach a specific reproducible number of developed leaves (Figure 30, Picture 1). Secondly, the test solution is sprayed on the seedlings by an automatic spray chamber that equally apportions the product (Figure 30, Pictures 2 and 3). Thirdly, after the mist has dried on the leaves, the pathogen is inoculated manually with a spray gun (Figure 30, Picture 4). Fourthly, the seedlings are incubated under semi-controlled conditions (Figure 30, Picture 5). Finally, the disease severity is scored manually on each leaf according to visual diagrams. Figure 31 and Figure 32 show examples of grapevine and apple seedlings, respectively. The automatic spray chamber is also utilised to simulate rainfall, which tests the ability of the product to remain on the leaves (Figure 30, Picture 3). For each tested product and references (blank, untreated, and copper control), three sets of six seedlings are employed. This procedure is described specifically in Chapters 2.4.1, 2.4.2, 2.4.3, and 2.4.4.

During seedling testing, the potential phytotoxicity of the sample is analysed. Phytotoxicity is defined as the capacity of a compound to cause temporary or long lasting damage to plants. The aspect that is observable on seedling testing is an eventual discoloration that could lead to necrosis [190].

Figure 30: Activity testing on seedlings under semi-controlled conditions.
Pictures: FiBL

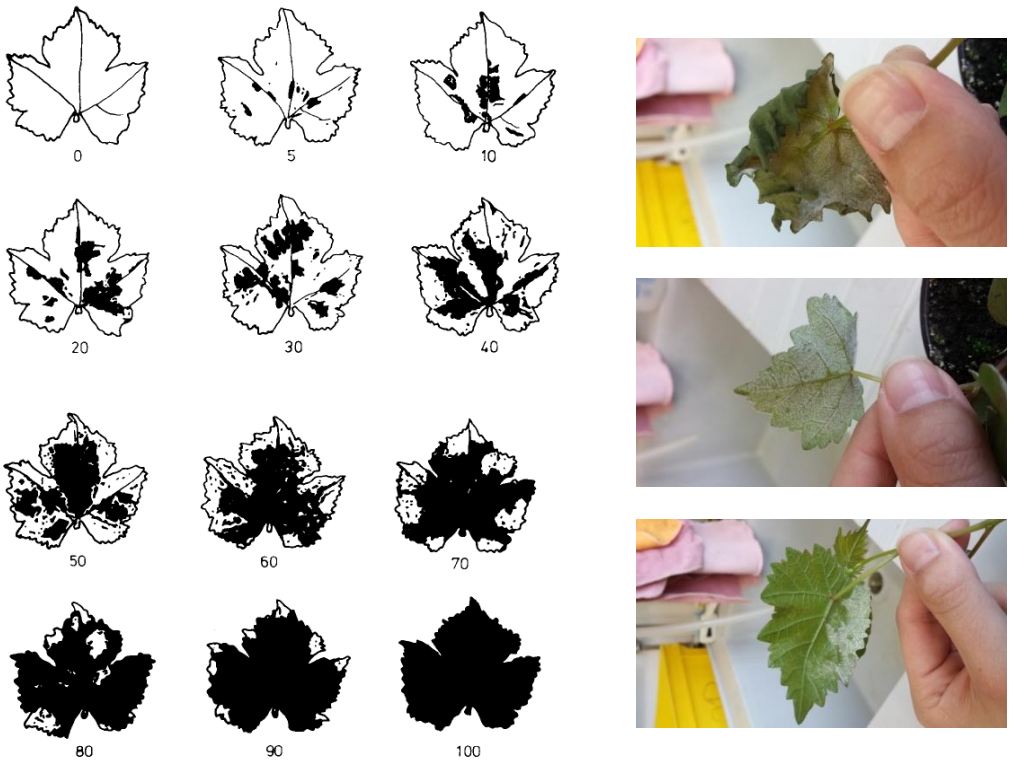

Figure 31: Visual diagram to assess the affected lower surface of grapevine leaves by *Plasmopara viticola* (Reproduced from [191]) and illustrations of the manual scoring (Pictures: J. Ramseyer).

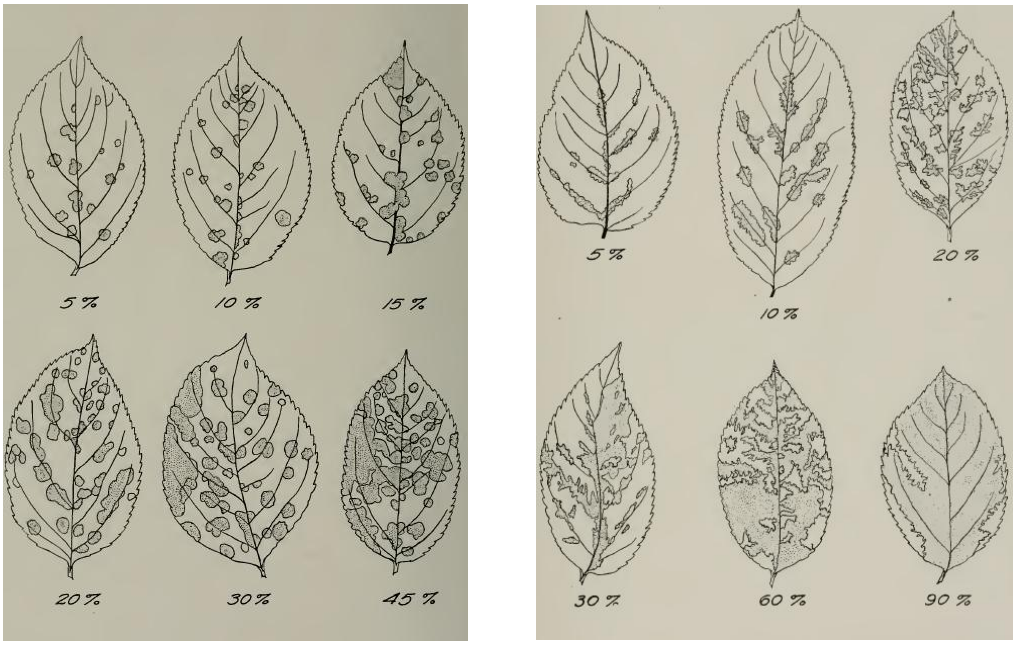

Figure 32: Visual diagram to assess the affected surface of apple leaves by *Venturia inaequalis* with separated (left) or diffuse (right) scab lesions.
Reproduced from [192]

2.3.4. Field trials

If promising results are obtained on seedlings, testing is upscaled to field conditions. This is performed on vineyards against *P. viticola* and apple orchards against *V. inaequalis* (Figure 33). The extract formulation is optimised to enhance the rain fastness, the solubility, and the stability of sensitive compounds.

Figure 33: FiBL vineyards (left) and apple orchards (right) for testing.
Pictures: FiBL

Specific guidelines are provided for field trials by the European and Mediterranean Plant Protection Organization (EPPO) [190, 191, 193, 194]. The trials should be performed on productive vineyards or orchards with natural infection of the pathogens, on the same susceptible cultivar, and on trees of the same age. The local cultural practices should be applied and all the plots have to be under uniform cultural conditions (for example soil type, fertilisation, etc.). The climate and topography should be homogeneous and favourable for the pathogen. The trials have to be repeated in different regions with different climatic conditions and during several consecutive seasons. Each trial has to include a reference product and an untreated control. Plot size is also regulated (e.g. apples: at least four trees and grapevines: the number of plants necessary for at least 100 bunches). At least four plot replicates are required. The meteorological data (temperature and precipitation) is recorded during the complete process. The frequency of treatment application is determined by a Decision Support System that determines the risk of infection according to different parameters [191, 193].

Assessment of disease on *Vitis vinifera* is performed on 100 leaves of each plot, the infected surface is determined as shown on Figure 31 in Chapter 2.3.3. Bunches are also

assessed, 100 bunches of each plot [191]. On *Malus domestica*, at least 200 leaves of long shoots and rosettes, or all the leaves of 20 long shoots per plot should be assessed according to Figure 32 in Chapter 2.3.3. Thereafter, 100 fruits per plot should be picked equally on each tree [193]. The first observation should be done when the first symptoms appears on the untreated control, and several ones throughout the season until harvest [191, 193].

During these procedures, potential phytotoxicity should also be followed further than the only eventual discoloration that could be noticed on seedlings. An impact may be observed on the development cycle, such as delay or inhibition of growth, flowering, fruiting, and ripening. The crop can then be thinned by failure of emergence or growing plant death. The different parts may also be deformed and finally affecting the quality and quantity of the harvest yield. Other aspects have to be taken into account such as the effects on other pests and non-target organisms [190, 191, 193].

At FiBL, the vineyard consists of two sensitive *Vitis vinifera* cultivars: “Müller-Thurgau (“Riesling x Sylvaner”) and “Chasselas” (“Gutedel”). A number of 288 plants per variety were planted in 1997. The distribution of the treatments is randomised. Figure 34 represents an example of nine different tested products, including an untreated control and a copper reference, applied on four replicates each consisting of 16 grapevine plants.

The FiBL apple orchard was planted in 2007 and includes 120 apple trees of the susceptible variety “Pinova”. Six randomised treatments can be applied in four replicates each consisting of five trees as shown on Figure 35.

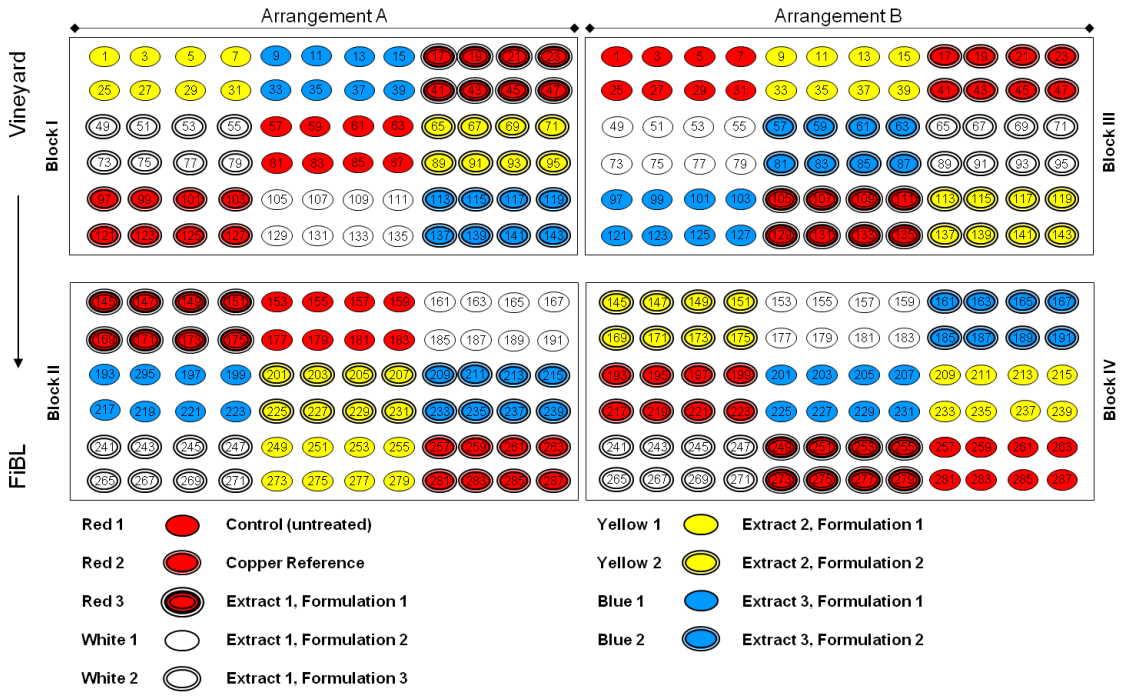

Figure 34: Example of randomised treatment distribution on the vineyard. Each treatment counts four replicates of 16 plants. The two cultivars are organised in a row mixture, one oval dot represents two plants: upper row with odd number are "Müller-Thurgau" and the lower row with even number (not shown) are "Chasselas". The arrow on the left side indicates the direction of the yard: vineyard being the top part going towards FiBL buildings (lower part).

Adapted from FiBL methods

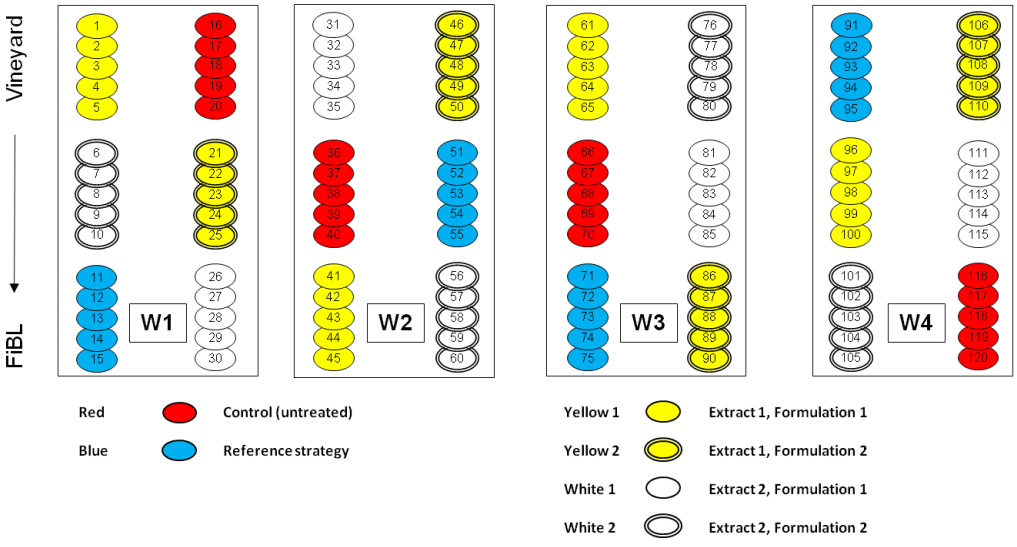

Figure 35: Example of randomised treatment distribution on apple orchard. Each treatment counts four replicates of five apple trees "Pinova". The arrow on the left side indicates the direction on the yard: vineyard being the top part going towards FiBL buildings (lower part).

Adapted from FiBL methods

2.4. Investigated plants

2.4.1. *Juncus effusus*

The genus *Juncus* (Juncaceae) comprises approximately 300 species of perennial or annual herbs, spread in Europe, South Pacific area, and Australia. The genus is rare in the tropics [195, 196]. The name *Juncus* derived from the latin word "*iuncus*" meaning rush [197].

The species *Juncus effusus* (Figure 36), named common rush, matting rush, or soft rush, was described by Carl Linnaeus (L.) in 1753. The species is native in Europe, Asia, Africa, North America, and South America. Moreover, it has been naturalised in Australia, Madagascar, and in various oceanic islands [198]. It grows from 200 to 3400 m altitude in moist or wet areas like forest margins, morasses, lake margins, and river banks [198-200]. *J. effusus* is a perennial rush with a thick rhizome. The green stems of 1 to 4 mm in diameter and 25 to 90 tall are densely tufted, terete, striate, and have a continuous medulla (Figure 37). The reddish-brown leaves of 2 to 22 cm long are sheathing the stems. Pale brown many-flowers inflorescences appear pseudo-lateral at about one-fifth from the stem apex, lax or condensed into a round head. The perianth size is about 2.0 to 2.7 mm long and 0.8 mm wide.

Figure 36: *Juncus effusus*.
Picture: L.-M. Landry

Figure 37: *Juncus effusus* medulla.
Picture: J. Ramseyer

Fruits are brown, ovoid to oblong capsules with approximately the length of the perianth [198-200]. The word "*effusus*" comes from the Latin verb "*effundere* – to pour" and refers to the inflorescences by meaning "let loose, free flying, fluttering" [197].

J. effusus has various applications in traditional medicine. Decoctions are used by the Cherokee Indians to induce vomiting and "to dislodge spoiled saliva". It is also given as an infusion to babies to prevent lameness, and utilised as a wash to strengthen them [201]. Some populations of Indian Ocean Islands use a decoction of the rhizome against stomach aches, dysentery and to stop bleedings. Furthermore, the decoction of the rhizome is applied as a bath to treat haemorrhoids in La Réunion [202]. Finally, the pith (Figure 37) is used in Traditional Chinese Medicine under the name "dengxincao" as sedative, antipyretic, detumescent, diuretic agent, and to treat oral ulceration [199, 203, 204].

The species *J. effusus* was phytochemically investigated and several types of compounds have been isolated (Figure 38) including phenanthrenes (**1** and **2**) [205], tetrahydropyrene glycosides [206], flavonoids [207-209], steroids (**3**) [208, 210], cycloartane triterpene glycosides/aglycones (**4**) [209, 211, 212], and phenolic acid derivatives (**5**) [207, 213]. Phenanthrenes are the major constituents of *J. effusus* medulla [203, 205, 214]. This compound class is relatively infrequent, and most of phenanthrenes were isolated from *Juncus* spp. An important number of them have also been reported in Orchidaceae family, especially in *Bletilla striata* and *Bulbophyllum vaginatum*. Their biosynthesis occurs seemingly through oxidative coupling of the aromatic rings of stilbene precursors [205].

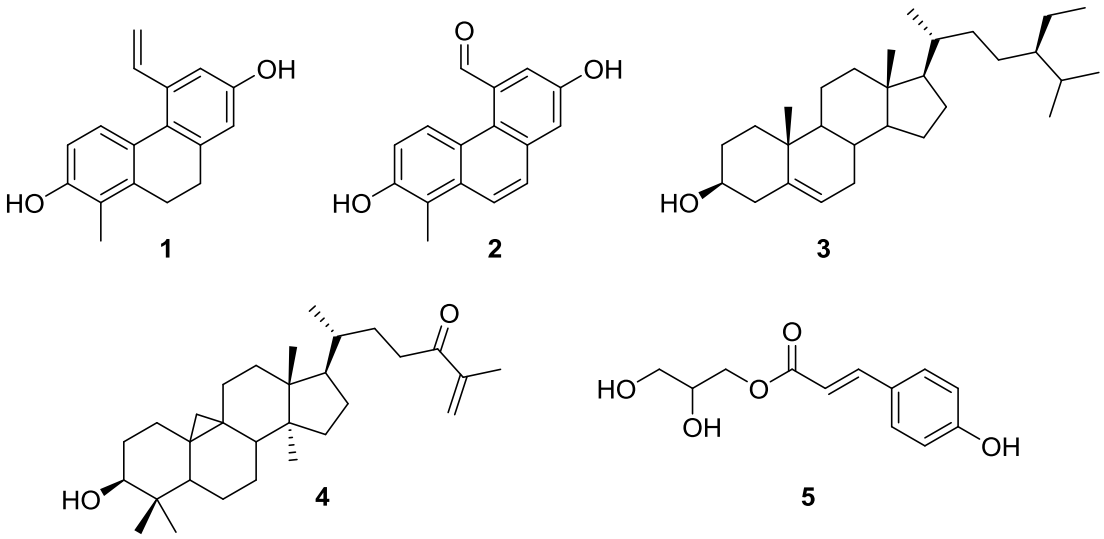

Figure 38: Selected compounds isolated from *Juncus effusus*: phenanthrenes (**1**: effusol, **2**: dehydroeffusal), steroid (**3**: *β*-sitosterol), cycloartane triterpene (**4**), and a phenolic acid derivative (**5**).

Phenanthrenes have a wide range of biological activities reported: cytotoxicity, anti-tumoral, antifungal, antibacterial, antiviral, spasmolytic, antiallergic, anti-inflammatory, anti-platelet aggregation, phototoxic, antioxidant, and antialgal on *Selenastrum capricornutum* [204, 205]. Besides, effusol and juncusol showed sedative and anxiolytic effects on mice in comparison with diazepam as a positive control [215]. These compounds are also photosensitizers. Their antimicrobial activity is enhanced under light [216]. Furthermore, phenanthrenes showed activity against *Agrobacterium tumefaciens*, a phytopathogenic bacterium inoculated on potato discs. This test is used as a substitute of some *in vivo* antileukemic activity assays [217, 218].

2.4.2. *Styrax tonkinensis*, *Styrax benzoin*, and *Styrax paralleloneurum*

The genus *Styrax* (Styracaceae) counts around 120 species of evergreen, resin-producing trees located in Mediterranean region, Southeast Asia, Malaysia, and tropical America (Figure 39) [196, 219-221]. Several species are exploited for their resin [222], a pathological secretion of the tree provoked after performing incisions in the trunk bark with a specific scheme (Figure 39). This exudate is called benzoin [219, 221, 223-228]. The two main types of benzoins, Siam benzoin (Figure 40) and Sumatra benzoin (Figure 41), are produced in Southeast Asia [220, 229]. Siam benzoin is extracted from *Styrax tonkinensis* in Thailand, Laos, Vietnam, Cambodia, and southern China mainly at an altitude between 150 to 2100 m. Sumatra benzoin is tapped from *Styrax paralleloneurum* and *Styrax benzoin* mainly in North Sumatra. The trees are also native in Borneo, Java, and the Malay Peninsula [219, 220, 223, 226, 229, 230]. *S. benzoin* grows at an altitude of 100 to 700 m on flat lands with good drainage. In contrast, *S. paralleloneurum* grows at 800 to 1700 m on rugged land in primary forest on clayey and rich soils, or in old secondary forest [222]. In 2002, around 70 tons of Siam benzoin and 4’000 tons of Sumatra benzoin were produced [220].

Figure 39: *Styrax* sp. tree with illustration of benzoin production on the right.
Picture: www.taliaessenze.com

Figure 40: Siam benzoin.
Picture: J. Ramseyer

Figure 41: Sumatra benzoin.
Picture: J. Ramseyer

S. tonkinensis was described by William Grant Craib and Carl Hartwich (Craib ex Hartwich) in 1913 [231]. "*Tonkinensis*" derives from the word "tonkinense", that means "native of Northern Vietnam"; Tonkin being the northern region of Vietnam [197, 223, 227]. The tree *S. tonkinensis* grows up to 25 m tall and the trunk to 30 cm in diameter with chocolate brown bark. The branching is light and more upright toward the top. The leaves are alternate, simple, ovate to elliptical, 4.5 to 10 cm long and 2.6 to 5 cm wide. The upper surface of the leaf is tough, dark green, leathery on the upper surface, and hairy, whitish green, very rough on the lower surface. Inflorescences are up to 18 cm long in double racemes appearing axils of the leaves or at the terminal part of the branch. They comprise many small, fragrant, white, flowers of 12 to 15 mm long with a 8 to 12 mm long corolla covered with yellowish hair on the outside and whitish hair in the inside. The fruit is ovoid, 10 to 12 mm long, 5 to 7 mm wide, and coated with greyish hair [221, 223].

S. benzoin was described by Jonas Dryander (Dryand.) in 1787 [232]. It grows up to 34 m tall and 100 cm in diameter. The bark is wine red and the wood white. Leaves are oval to oblong reaching 6 to 13.5 cm long and 2.5 to 6 cm wide with the lower face covered by greyish hair. The fragrant inflorescences are paniculated, 6 to 10 cm long, terminal and axial. The corolla lobes are 7 to 12 mm long. The mature indehiscent fruits are globose or oblong, and 8 to 12 mm in diameter [196, 220-222].

Janet Russell Perkins (Perk.) originally described *S. paralleloneurum* in 1902 [233]. This tree grows up to 35 m tall and 60 cm trunk diameter. The leaves are oblong or oblong-lanceolate with a size of 6 to 16 cm long and 2.5 to 3 cm wide. Their upper surface is glabrous, their lower surface is covered by thick whitish hair, but less dense than on *S. benzoin*. Inflorescences are mutlifloral, cymose, multibranched, and flowers are around 13 mm with the corolla covered with yellowish hair on the outside. The fruits are oblong-globose to elliptical with a diameter of 5 to 9 mm [221, 222, 233].

Siam benzoin resin tears or blocks are yellowish-brown or reddish-brown on the outside surface. The colour changes to darker shades by the exposure to air. It breaks easily; freshly broken, the inside is smooth, opaque, and milky white. By application of heat, the exudate becomes soft and melts. The resin possesses an aromatic pleasant smell, sweet-balsamic with vanilla notes. It gets plastic when chewed, taste sweet at the beginning, and then slightly pungent [219, 221, 223, 225, 227, 234-237]. On the other hand, Sumatra benzoin tears are brittle, opaque, reddish or whitish. They melt easily when heated, have a pleasant balsamic smell with a touch of vanilla, and taste somewhat acrid [219-221, 227, 235-237].

Many applications of benzoin are reported. It is broadly used in different religions for fumigations during traditional or religious rituals. In the same manner, some cigarettes are flavoured with that resin. It is also a major ingredient of Armenian incense paper, because it has the power to annihilate foetid odours [220, 222, 224, 238, 239]. Moreover, Siam benzoin is exploited in perfume industry for his outstanding fixative properties. In cosmetic, the balsam is used as anti-oxidative and preservative agent. Sumatra benzoin also, but in a smaller extent, because it is considered as an inferior product [222, 223, 226, 229, 230, 239]. Furthermore, Siam benzoin is classified as a natural flavour and is utilised in most types of food (beverages, desserts, candies, chewing-gums, gelatines, puddings, frozen dairies, baked goods...). It is also exploited in food industry as anticaking, coating, glazing, texturizing, and preservative agent. In addition, it has the properties to fix, support, and stabilise the flavours [224, 226, 240, 241].

Siam benzoin is a drug of Traditional Chinese Medicine called “anxixiang”. Indications are impairment of consciousness, rheumatism, cough, cold, abdominal ache, chest pain (heart burn), and externally for curing skin ulcers [223, 225, 242]. Besides, tincture of benzoin, called commonly “Friar’s balsam”, and other preparations are used as expectorant for bronchitis, as stimulant, as diuretic, as antiseptic, and as styptic on small cuts. In dentistry, herpetic lesions and gum inflammation are treated with benzoin [219, 220, 226, 227, 229, 235, 243]. Moreover, a milky alcoholic solution of benzoin, called Virgin’s milk, has been employed for feminine hygiene [224].

Sumatra and/or Siam benzoin are or have been part of many Pharmacopoeia including those of Europe, Great-Britain, Switzerland, Japan, Brazil, [234, 236, 237, 244, 245]. A property of benzoin resin is a relief effect on nervous tension and headaches which therefore helps for intellectual concentration [239]. Furthermore, Sumatra benzoin and Siam benzoin have antioxidant and immunostimulating properties [244].

The major constituents of Siam benzoin (Figure 42) are the ester of benzoic acid coniferyl benzoate (15 to 60%) (**1**), benzoic acid itself (15 to 45%), vanillin (**2**) (<5%), benzyl benzoate (<2%), and *p*-coumaryl benzoate. Vanillin is an oxidation product of coniferyl alcohol. Other compounds are triterpenoids (e.g. siaresinolic (**3**) and sumaresinolic (**4**) acids), and morinol derivatives. Cinnamic acid and its esters are not present [219, 221, 225, 227-230, 234-237, 240, 241, 244, 246-248]. In contrast, Sumatra benzoin (Figure 42) contains mainly esters of cinnamic acid (e.g. *p*-coumaryl cinnamate (**5**) and cinnamyl cinnamate), and free cinnamic acid. Some benzoic acid and esters thereof are also found, but about two times less than the amount of cinnamic compounds. In addition, some triterpenoid acids are also found (siaresinolic (**3**) and sumaresinolic (**4**) acids), the lignan pinoresinol, and vanillin (**2**) [219, 227-230, 235-237, 241, 244, 248, 249]. It has to be mentioned that the reported chemical composition of those two benzoin types is sometimes contradictory between references.

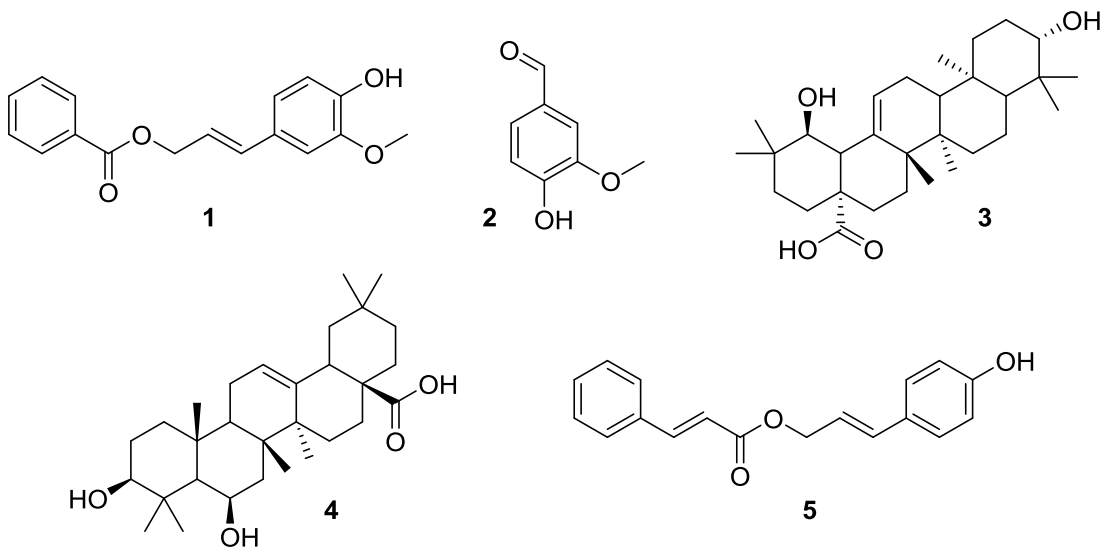

Figure 42: Selected compounds isolated from Siam benzoin and/or Sumatra benzoin: coniferyl benzoate (**1**), vanillin (**2**), siaresinolic acid (**3**), sumaresinolic acid (**4**), and *p*-coumaryl cinnamate (**5**).

Pinoresinol, lignan isolated from Sumatra benzoin, shows antioxidant and antihypertensive activity. Besides, triterpenes, present in both benzoin types, exhibit cytotoxicity against tumour cell lines [244], and anti-proliferative/differentiation effects on leukaemia cells [247]. Finally, benzyl benzoate, a minor compound of Siam benzoin, shows *in vitro* antifungal activity against the phytopathogens *Pyricularia oryzae*, *Rhizoctonia solani*, and *Sclerotium rolfsii* [250].

2.4.3. *Magnolia officinalis*

The genus *Magnolia* (Magnoliaceae) counts about 120 species distributed in America and in tropical South East Asia [251]. The Magnoliaceae are an ancient family that exists since the Tertiary Period [252]. The name *Magnolia* was given by the French botanist Charles Plumier in 1703 as tribute to an another French botanist Pierre Magnol, who worked on this family [197].

Magnolia officinalis (Figure 43), described by Alfred Rehder and Ernest Henry Wilson in 1907 (Rehder & Wilson), is native of China and is growing in subtropical broadleaf forest at an altitude of 300 to 2000 m. It is a deciduous tree between 6 to 15 m tall. The leaves are green with a long stalk, rounded tip, and measure 35 to 45 cm long, and up to 20 cm wide. Flowers, from April to May, are white, fragrant, and cup-shaped (15 to 20 cm). The fruits, appearing in October, are elongated and ovoid, from 10 to 12 cm long and a diameter of 5.5

to 6.0 cm. Seeds are obovoid (1.2 cm long, 1.0 cm wide) [251, 253, 254]. The term "*officinalis*" comes from the Latin word "*officina*" meaning "factory", and by extent "the room, in which the formulation and dispensing of medicines is performed" [197].

M. officinalis dried bark (Figure 44) is used in Traditional Chinese Medicine. Called "houpo", the bark has a pungent, bitter, and "warm" taste and is tonic, carminative, stomachic, and quieting. The drug is prescribed to treat diarrhoea, flatulencies, amenorrhea, fever, asthma, productive cough, and various gastro-intestinal difficulties [225, 255-257].

Figure 43: *Magnolia officinalis*.
Pictures: C. Basset

Figure 44: *Magnolia officinalis* dried bark.
Picture: J. Ramseyer

The main constituents of the bark (Figure 45) are monoterpenyl-lignans (e.g. piperitylmagnolol (**1**), piperitylhonokiol, and bornylmagnolol), lignans (e.g. honokiol (**2**), magnolol (**3**), magnaldehydes B and C, and magnolignans A-E), norlignans (e.g. magnaldehydes D and E, and magnatriol B), and dilignans (e.g. magnolignans F-I (**4**)). It also

contains some quaternary alkaloids (e.g. magnoflorine (**5**), magnocurarine, and tembetarine) The bark contains large amount of honokiol and magnolol: 0.3 to 4.6% and 2 to 11%, respectively [251, 258-260].

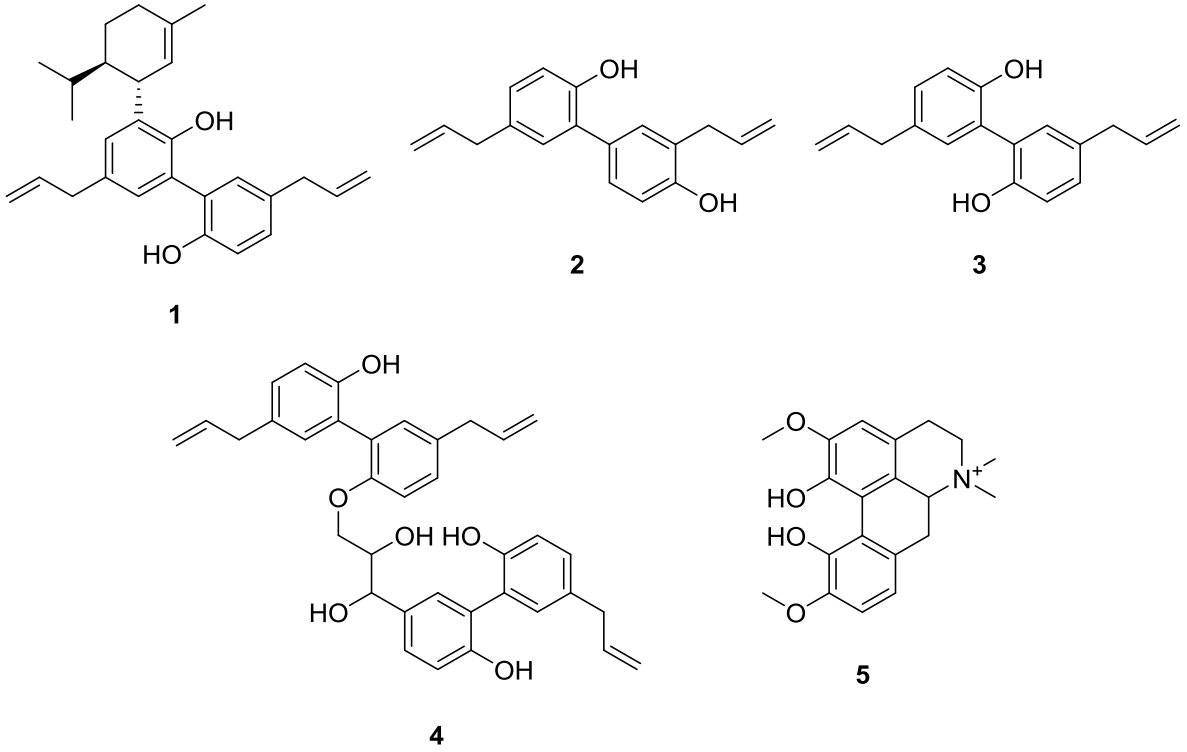

Figure 45: Selected compounds isolated from *Magnolia officinalis* bark: piperitylmagnolol (**1**), honokiol (**2**), magnolol (**3**), magnolignan F (**4**), and magnoflorine (**5**).

Lignans are widely distributed through the plant kingdom and found in up to 70 botanical families. The compounds are considered to be one of the earliest forms of defence in vascular plants against predators and pathogens. The complexity of the structures increase with the evolution [261]. Lignans are synthesised through the shikimate pathway, and are usually dimers of phenylpropanoid units [71, 261]. This class of compounds exerts a broad spectrum of pharmacological activities, such as oestrogenic (phytoestrogen), anticancer, antiviral, antifungal, antibacterial, parasiticidal, insecticidal effects, and modification of the cardiovascular function by different mechanisms [261].

Honokiol and magnolol also possess a wide range of biological properties, comprising antiemetic, anticonvulsant, anti-inflammatory, anti-allergic, anti-asthmatic, antioxidant, antiviral, antibacterial, and antifungal activities. Furthermore, they exert cytotoxic, anti-mutagenic, and anti-tumour properties. Moreover, they reduce the blood pressure, increase heart rate, have anti-arrhythmic properties, and inhibit the blood clotting. Finally, depressant

effect on central nervous system (anxiolytic), neuroprotective properties, prevention of peptic ulcer, and muscle relaxation were also observed [251, 252, 255-257, 262-276].

In agriculture studies, bark extracts of two *Magnolia* species (*M. officinalis* and *M. obovata*) and isolated compounds (mainly magnolol and honokiol) show *in vitro* activity against some plant pathogenic and wood decay fungi [277-279]. Under controlled conditions, a formulated extract of *M. obovata* as a wettable powder exert *in vivo* activity against rice blast, tomato late blight, wheat leaf rust, barley powdery mildew, and red pepper anthracnose [278]. Furthermore, a wettable powder of *M. officinalis* bark extract decreases rust diseases of *Perilla* and *Zoysia* grass under field conditions. A patent was deposited in South Korea in 2009 for a *Magnolia* sp. extract as a control for plant disease [280].

2.4.4. *Verbesina lanata*

Commonly known as crownbeard, the genus *Verbesina* (Asteraceae) comprises more than 300 species of shrubs, herbs, and trees mainly distributed in from Eastern Canada to Central Argentina which are characterised by numerous bright yellow flowers [195, 281, 282]. The name *Verbesina* comes from the comparison of *Verbesina encelioides* white tomentose stalks with sheep wool, in Latin "*verbecina lana*" [197].

Verbesina lanata (Figure 46), described by B. L. Robinson and J. M. Greenman (B. L. Rob. & Greenm.) in 1899, is growing in Central America. The species is a shrub or tree that grows up to 4 meter tall. The twigs are stout and tomentose covered by yellowish brown hairs, hence the name "*lanata*" which means in Latin covered by wool in Latin [197]. The leaves are alternate, elliptic or lanceolate, acuminate at both ends, glabrescent above, and pilose beneath. Inflorescence bears a large many-headed panicle with yellow corolla [283, 284].

A few species of *Verbesina* have reported uses in traditional medicine [282]. *V. negrensis* Steyerm. and *V. turbacensis* H. B. K. are used for their wound healing properties [282, 285, 286]. Besides, *V. turbacensis* is also employed to treat gastro-intestinal disorders [287]. *V. virginica* L. is utilised by the Chickasaw, Choctaw, and Seminole Indians of North

Figure 46: *Verbesina lanata*.
Picture: A. Espinosa, CIFLORPAN, Panama

America mainly as laxative, emetic, diuretic, and anti-rheumatic drug. Other applications by the Chickasaw are against leukorrhea, uterine weakness, and venereal diseases [288-290]. The species *V. encelioides* (Cav.) Benth. & Hook. Fil ex Gray is used by the Navajo, Hopi, and Zuni Indians of North America mostly against fever, itch after spider bite, and as emetic drug [201, 291-295].

Verbesina lanata has not been phytochemically investigated yet. According to Mora *et al*. [282], about one eighth of the *Verbesina* species have been studied up to 2013 and only a few of them are biologically investigated. Antibacterial, fungicidal, antiviral, antihypertensive, and cytotoxic activities have been reported [282, 296]. Around 200 compounds have been isolated and characterised [282]. The most abundant class of substances are terpenoids (75.4%), followed by various aromatics compounds (12.8%), flavonoids (3.5%), guanidine alkaloids (2.9%), acetylenes (2.5%), and miscellaneous compounds (2.9%). Sesquiterpenes are the largest represented class of terpenoids (79%). Among the isolated compounds, the main group of constituents are eudesmane cinnamates (26.5%) [282]. Eudesmanes, with many different oxygenation and cleavage patterns, are characteristic secondary metabolites of the Asteraceae family [297].

Sesquiterpenes are usually synthesised through the mevalonate pathway starting from farnesyl pyrophosphate (FPP) [71]. The eudesmane skeleton contains a *trans*-decaline ring system substituted by two methyl and one isopropyl groups [297]. Eudesmanes found in the genus *Verbesina* are highly oxygenated and a large number of them contain a cinnamate moiety as substituent at position 6 or 4 (Figure 47, **1**-**6**) [282].

Numerous studies have focussed on this type of compound for the last two decades, especially because of their wide range of biological activities which include antibacterial, cytotoxic on tumor cell-lines, antifungal, plant-growth regulation, and insect anti-feeding properties [297]. The antifungal activity of eudesmanes was protected by a patent in the United States in 1999. This patent comprises the medical and agricultural uses [298].

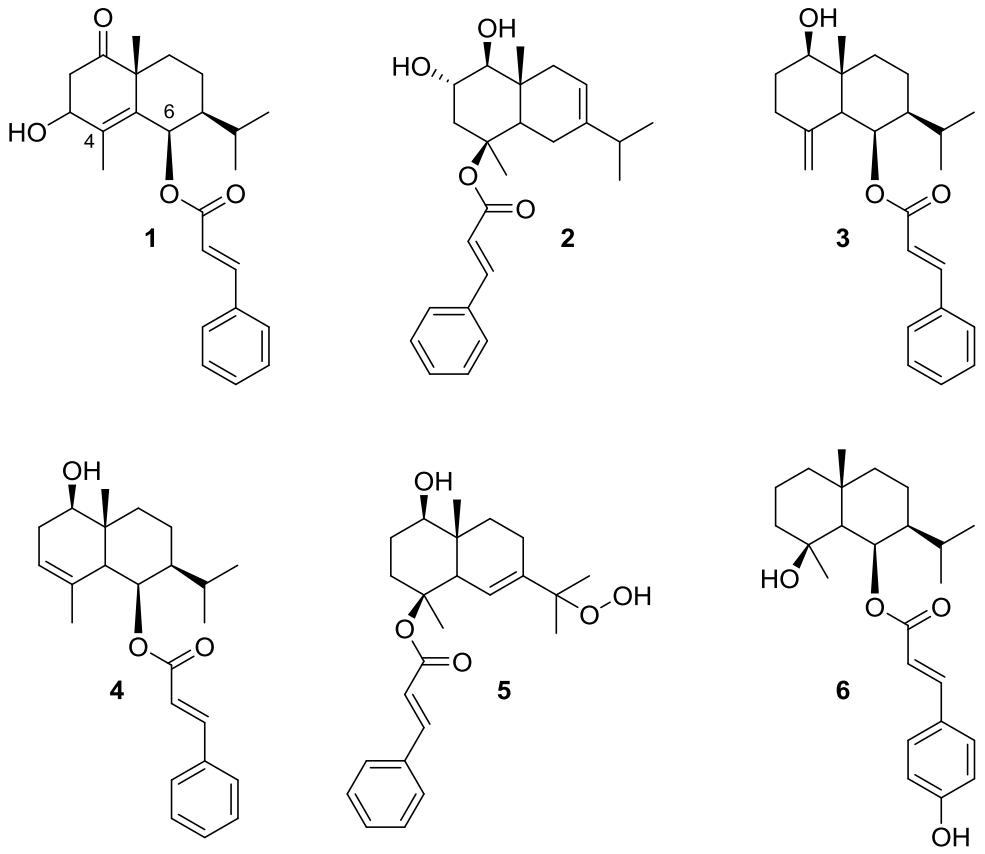

Figure 47: Selected eudesmane sesquiterpenes isolated from *Verbesina* genus (**1**-**6**).

2.4.5. *Iryanthera megistocarpa*

The genus *Iryanthera* (Myristicaceae) comprises about 20 species of dioecious or monoecious trees and shrubs distributed from the northern half of South America to Panama [195, 299, 300].

Iryanthera megistocarpa (Figure 48) was described by Alvin Howard Gentry (A. H. Gentry) in 1975. The discovery was in Panama on the road from El Llano to Carti in a tropical wet forest at an altitude of 330 – 370 m. The species is endemic to Panama, and a few trees have also been found in Colombia. *I. megistocarpa* is a dioecious tree of at least 15 m tall. The leaves are narrowly elliptic, with a more or less rounded base. They measure 11 to 17 cm long and 4.2 to 7 cm wide. Fruits are ellipsoid to subglobose (4 to 5 cm long, and 3.5 to 4 cm wide) with a wrinkle-rugose, and more or less glabrous surface [301, 302].

Iryanthera spp. have reported uses in traditional medicine [303]. The resin-like exsudate of *I. grandis* Ducke, *I. juruensis* Warb., and *I. paraensis* Huber is employed against skin fungus; *I. paraensis* is used as well against mites. *I. juruensis* possesses further traditional

applications: the latex is exploited against diarrhoea and difficult menstruations, the leaves against vomiting and diarrhoea, and mashed bark is applied to heal skin hurts [304]. Besides, *I. tessmannii* Markgr. bark is used against diarrhoea [304]. Furthermore, the bark of *I. lancifolia* Ducke is applied against skin infection [304]. Moreover, the Puinav Colombian Indians apply *I. ulei* (Benth.) Warb. crushed leaves to clean infected wounds [305]. The Uitoto community consumed the bark latex of *I. ulei* mixed with warm water to heal food poisoning caused by bad fish or meat consumption [305]. This community employs a bark aqueous extract of an another species, *I. tricornis* Ducke, internally against measles [306]. *I. tricornis* is also used to heal burns and infected cuts [305]. Finally, a decoction of *I. megistophylla* A. C. Sm. bark is utilised externally by Afro-Colombian community to treat cutaneous leishmaniasis [306].

The species *I. megistocarpa* has not been phytochemically investigated yet. Approximately ten *Iryanthera* species have been studied up to now. The major classes of isolated compounds are butanolides, diarylpropans, dihydrochalcones, flavonoids, γ-lactones, lignans, tocotrienols, and flavonolignans [303, 307-332].

Only a few species have been biologically investigated. One of them is *I. tricornis* extract with antimicrobial properties [306]. A second one is the polar fraction *I. ulei* Warb extract with antioxidant properties [320]. Another one is *I. megistophylla* showing several *in vitro* activities including (i) antiviral against Herpes Simplex Virus due to cinchonains, (ii) antiviral against Potato Virus X due to iryantherin K, cinchonains, and procyanidin B-2, (iii) antifungal against *Candida albicans* due to cinchonains and procyanidin B-2, (iiii) antibacterial against *Staphylococcus aureus* due to iryantherin K and procyanidin B-2, and (iiiii) anti-acetylcholinesterase due to megislignan and iryantherin K [306, 316]. In addition, *I. laevis* bark is active against Gram positive bacteria [330].

Furthermore, the dibenzylbutane lignans isolated from *I. lancifolia* show oestrogenic properties [315]. Two neolignans of *I. juruensis* arils have antileshmanial activity against the promastigote form of *Leishmania amazonensis* [324]. A hexane extract of the same species seeds inhibits β-carotene oxidation, lipid peroxidation, and cyclooxygenase 1 and 2 [322, 323]. In this

Figure 48: *Iryanthera megistocarpa*. Picture: A. Gentry

case, β-carotene oxidation is hindered by tocotrienols and lignans [322]. The compounds responsible for the cyclooxygenase inhibition are flavones and sargaquinoic acid [323], while ω-arylalkanoic acids, sargaquinoic acid, and flavones inhibit lipid peroxidation [323].

Flavonolignans isolated from *Iryanthera* spp. mainly consist of a lignan coupled by oxidation to the cycle A of a dihydrochalcone and are named iryantherins (Figure 49) [312, 328].

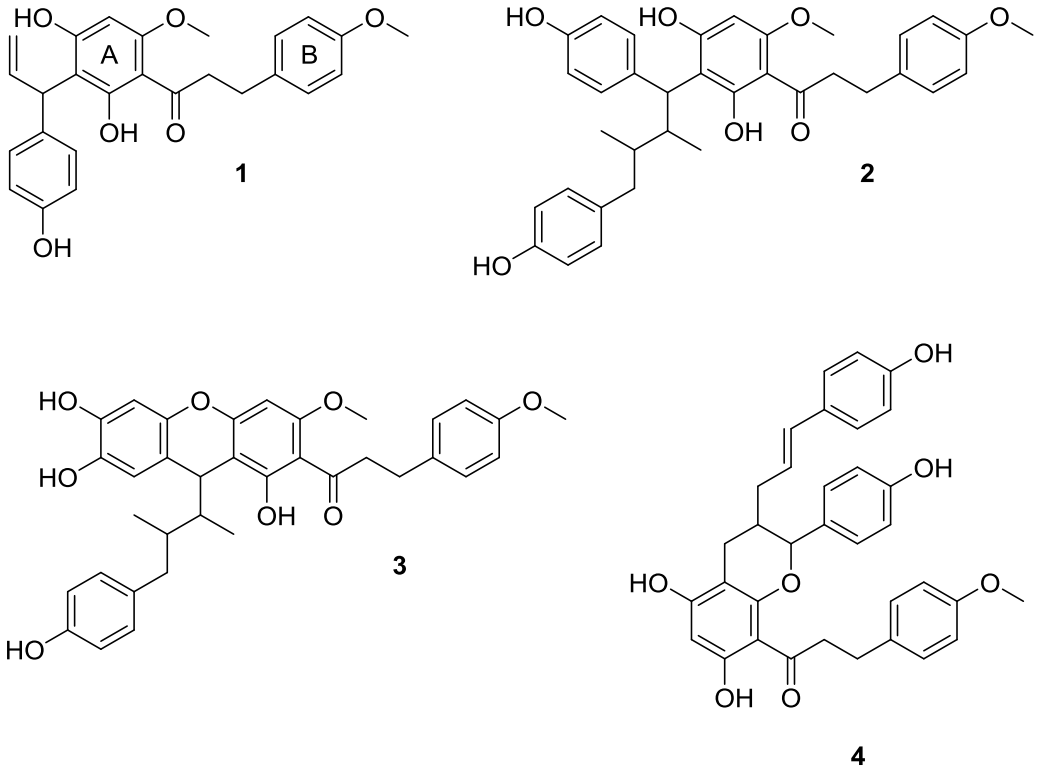

Figure 49: Selected flavonolignans isolated from *Iryanthera* genus: Iryantherin A (**1**), iryantherin B (**2**), iryantherin C (**3**), iryantherin D (**4**).

This compound class has not been widely investigated for biological properties. One study shows their antioxidant activity [314]. Additionally, iryantherin K is antibacterial against *Staphylococcus aureus*, has anti-acetylcholinesterase properties, and shows antiviral activities against the phytopathogenic Potato Virus X [316]. Finally, two dihydrochalcones (2',4'-dihydroxy-6'-methoxy-3,4-methylenedioxydihydrochalcone and 2',4'-dihydroxy-4,6'-dimethoxydihydrochalcone) are cytotoxic against tumor cell lines [333], show oestrogenic activity [315]. The second dihydrochalcone is anti-inflammatory [334] and active against Gram positive bacteria [330].

3. RESULTS AND DISCUSSION

3.1. Efficacy of a *Juncus effusus* Extract on Grapevine and Apple Plants against *Plasmopara viticola* and *Venturia inaequalis*, and Identification of the Major Active Constituent

Barbara Thuerig, Justine Ramseyer, Matthias Hamburger, Thomas Oberhänsli, Olivier Potterat, Hans-Jakob Schärer, and Lucius Tamm

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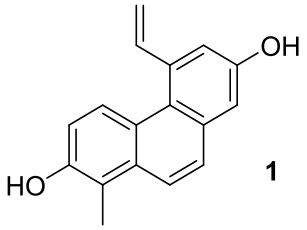

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An in-house library of plant and fungal extracts was screened *in vitro* for their inhibition against several plant pathogens. As one of the hits, the ethyl acetate extract of *Juncus effusus* L. (Juncaceae) medulla showed strong inhibitory activity against *Plasmopara viticola* (grapevine downy mildew), *Venturia inaequalis* (apple scab) and *Phytophthora infestans* (tomato and potato late blight). The major active constituent was identified as dehydroeffusol (**1**) by a procedure referred to as HPLC-based activity profiling which combines biological activity data with chemoanalytical information. Subsequent *in planta* assessment of the extract and dehydroeffusol revealed potent inhibition of the disease on grapevine and apple seedlings.

Extraction of plant material, HPLC-microfractionation, isolation of pure compound, quantification, preparation of Figures 1 and 2, writing the drafts of Chapter 2.1. and a part of Chapter 3.1. were my contributions to this publication.

Justine Fabienne Ramseyer

Research Article

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Efficacy of a *Juncus effusus* extract on grapevine and apple plants against *Plasmopara viticola* and *Venturia inaequalis*, and identification of the major active constituent†

Barbara Thuerig,[a*] Justine Ramseyer,[b] Matthias Hamburger,[b] Thomas Oberhänsli,[a] Olivier Potterat,[b] Hans-Jakob Schärer[a] and Lucius Tamm[a]

Abstract

BACKGROUND: There is growing demand to replace chemical pesticides with alternatives owing to concerns related to impacts on human health and the environment. Plant-derived plant protection products could provide sustainable and environmentally friendly alternatives to chemical products. The aim of this study was to identify plant and fungal extracts with so far unknown activity against important plant pathogens by *in vitro* screening of a library of more than 3000 extracts.

RESULTS: Several plant extracts with promising *in vitro* fungicidal activity ($MIC_{100} \leq 50\ \mu g\ mL^{-1}$) towards one or several of the investigated pathogens (*Venturia ineaqualis*, *Phytophthora infestans*, *Plasmopara viticola*) were identified by the screening. One of the hits, an ethyl acetate extract of *Juncus effusus* L. medulla, was further investigated, and dehydroeffusol (DHEF) was identified as its main active constituent. On susceptible grapevine and apple seedlings, efficacies of up to 100% were reached with the extract (EC_{50} 123 or 156 $\mu g\ mL^{-1}$) and with DHEF (EC_{50} 18 or 21 $\mu g\ mL^{-1}$) against *P. viticola* and *V. inaequalis* respectively.

CONCLUSIONS: Our results demonstrate that plants can provide promising alternatives for integrated and organic farming. *J. effusus* shows high efficacy at low concentrations and, as an abundant perennial species, is an interesting candidate for the development of a novel plant protection product.

Keywords: *Juncus effusus*; dehydroeffusol; plant extract; fungicide; downy mildew; apple scab

1 INTRODUCTION

Pests and diseases are a constant threat to agricultural production, and climate change is likely further to increase their occurrence, prevalence and impact. Yet, there is growing demand to replace chemical pesticides with alternatives owing to concerns related to impacts on human health and the environment.[1–6] The use of pesticides should therefore be reduced or avoided whenever possible. For example, copper is widely used to control devastating plant diseases such as grapevine downy mildew (*Plasmopara viticola*), potato and tomato late blight (*Phytophthora infestans*), apple scab (*Venturia inaequalis*) and a wide range of other plant pathogens.[7,8] However, copper should be replaced urgently, as it has an unfavourable ecotoxicological profile.[9] Control of pathogens by means of plant-derived plant protection products can be an effective, sustainable and environmentally friendly method for pest management in integrated pest management (IPM) and organic farming systems.[10] Extracts of selected plants, such as *Reynoutria sacchalinensis*,[11] *Glycyrrhiza glabra*,[12] *Salvia officinalis*,[13] *Inula viscosa*,[14] *Larrea divaricata*[15] and *Yucca schidigera*,[16,17] have been shown to be active against plant diseases. Yet, very few plant extracts have been developed for commercial use. At the time of writing, fennel oil (against powdery mildews and rust), lecithine (against powdery mildews), coconut potassium soap (against rainspot disease of apples) and laminarine (an alga-derived stimulator of natural defence mechanisms in plants) are registered in Switzerland,[8,18] while some of them are under review in the EU. Extract of *Reynoutria sacchalinensis* has been registered in Germany as a plant strengthener in the past.

The screening of botanical libraries has been widely used in pharmaceutical drug discovery, and this approach is also ideally suited to the discovery of plant extracts with activity against plant pathogens. The use of the database format for extract handling and new strategies combining high-performance liquid chromatography (HPLC) microfractionation with spectroscopic and bioactivity data enables prioritisation of hits and identification

* *Correspondence to: B Thuerig, Research Institute of Organic Agriculture FiBL, Ackerstrasse 113, CH-5070 Frick, Switzerland, E-mail: barbara.thuerig@fibl.org*

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a *Research Institute of Organic Agriculture FiBL, Frick, Switzerland*

b *Department of Pharmaceutical Biology, University of Basel, Basel, Switzerland*

of bioactive constituents at an early stage,[19] given that miniaturised bioassays are available for the screening. In the present study, a library containing more than 3000 extracts from approximately 800 plants and 100 fungi was screened for activity against some important plant pests (*Plasmopara viticola*, *Venturia inaequalis*, *Phytophthora infestans*) to identify natural extracts with so far unknown inhibitory activity.

One of the hits, an ethyl acetate extract of *Juncus effusus* L. medulla, was selected for further investigation. *Juncus effusus* is a perennial, nearly cosmopolitan species of the Juncaceae family growing in wet areas. It has long been used in traditional Chinese medicine as a sedative, anxiolytic, antipyretic and detumescence agent.[20,21] Many secondary metabolites have been reported from the species, including flavonoids,[22–24] phenolic acid derivatives,[22,25] coumaroyl glycerides,[24] cycloartane-type triterpenes,[26,27] steroids[24,28] and phenanthrene derivatives.[29] Hanawa *et al.*[30] showed antimicrobial activities of two compounds of *J. effusus* (juncusol and dehydroeffusol) against human pathogens (*Candida albicans*, *Staphylococcus aureus*, *Bacillus subtilis*), especially in the presence of UV light, and Della Greca *et al.*[31,32] reported activity against the green algal species *Selenastrum capricornutum*. However, no investigations have been reported up to now on the efficacy of *J. effusus* extracts against plant pathogens, besides an inhibitory effect of leaf homogenates against the cucumber pathogen *Colletotrichum lagenarium*.[33]

The aims of the present study were (i) to identify a plant extract with activity against *Plasmopara viticola*, *Venturia inaequalis* and *Phytophthora infestans* using an *in vitro* bioassay, (ii) to identify its active constituents and (iii) to determine the activity of the plant extract and the active compounds *on planta* against apple scab and downy mildew.

2 MATERIALS AND METHODS

2.1 Phytochemistry

2.1.1 Chemicals

Solvents and formic acid were obtained from Scharlau (Barcelona, Spain). For extraction, technical-grade solvents were used after redistillation. For high-performance liquid chromatography, HPLC-grade solvents were used. HPLC-grade water was obtained from a MilliQ water purification system (Merck Millipore, Darmstadt, Germany). Deuterated solvents for nuclear magnetic resonance (NMR) analysis were purchased from ARMAR Chemicals (Döttingen, Switzerland).

2.1.2 Plant extract library

An extract library consisting of 3077 extracts from approximately 800 plants and 100 fungi was used.[34] The extract library had been previously prepared by extraction of a few grams of plant material with the aid of pressurised liquid extraction (accelerated solvent extraction) on an ASE 200 instrument (Dionex, Sunnyvale, CA). Of each plant sample, three extracts were obtained using solvents of increasing polarity (typically petroleum ether or dichloromethane, followed by ethyl acetate and methanol). After extraction, extracts were dried, dissolved in DMSO at 10 mg mL^{-1} and transferred to 96-well storage plates, from where they were replicated into 96-well daughter plates for the *in vitro* screening.

2.1.3 Plant material

The medulla of the common rush (*Juncus effusus* L.) was purchased from Bollwerkapotheke AG in Bern, Switzerland. The plant material was imported from Kunming in China (Yunnan Baiyao Group Co., Ltd). A voucher specimen (No. 909) is kept at the Department of Pharmaceutical Biology, University of Basel, Switzerland.

2.1.4 Preparative extraction

To produce an extract for plant-pathogen bioassays, and for isolation of the active compound, the medulla of *J. effusus* was frozen with liquid nitrogen and milled with a mortar and pestle. The powdered material (183 g) was then mixed with sea sand (approximately 1 kg) (Carl Roth GmbH, Karlsruhe, Germany) and extracted at room temperature by percolation in a 40 cm × 10 cm i.d. column with 13 L of ethyl acetate. After evaporation under reduced pressure, 0.91 g of extract was obtained (yield 0.5%).

2.1.5 General procedures

Preparative HPLC was performed on a LC8A preparative liquid chromatograph consisting of an SCL-10VP controller, LC-8A binary pumps and a UV–vis SPD-M10A detector (Shimadzu, Kyoto, Japan), using a SunFire™ Prep C_{18} OBD column (5 µm, 150 × 30 mm i.d.; Waters, Milford, MA). Semi-preparative HPLC was performed on an Agilent 1100 Series system with a PDA detector (Santa Clara, CA) connected to a FC204 fraction collector (Gilson, Middleton, WI). Separations were carried out on a SunFire™ Prep C_{18} column (5 µm, 150 × 10 mm i.d.; Waters) equipped with a guard column (10 × 10 mm i.d.). Mass spectra were obtained on an Esquire 3000 Plus mass spectrometer equipped with an ESI source (Bruker Daltonics, Bremen, Germany) that was coupled to an Agilent 1100 Series HPLC system. NMR spectra were recorded on a 500 MHz Avance III™ spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a 1 mm TXI microprobe. Standard pulse sequences of the software package Topspin 3.0 were used.

2.1.6 HPLC microfractionation

Microfractionation was performed by semi-preparative HPLC. The mobile phase consisted of water with 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). A gradient of 5–100% B in 30 min was used, followed by isocratic conditions of 100% B for 5 min. The flow rate was 4.0 mL min^{-1}. The extract was dissolved in DMSO at a concentration of 50 mg mL^{-1}, centrifuged and filtered. Two injections of 200 µL were performed (20 mg of extract in total). Microfractions were collected every 90 s from 2 to 35 min (22 fractions per injection). After removal of the eluent in a Genevac EZ-2 evaporator (Stone Ridge, NY), the fractions were redissolved in 300 µL of methanol. The corresponding fractions obtained from the two separations were combined and dried. Before testing, the fractions were dissolved in 100 µL of DMSO.

2.1.7 Isolation of the active constituent

A portion of the ethyl acetate extract (271 mg) was separated by repeated injections into a preparative HPLC to afford a crude product (19.6 mg, R_t 21.7 min). The mobile phase consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). A gradient of 30–80% B in 30 min was used. The flow rate was 20 mL min^{-1}. Detection was at 254 nm. Final purification was achieved by semi-preparative HPLC. The mobile phase consisted of water with 0.1% formic acid (solvent

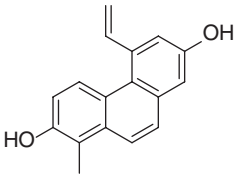

Figure 1. Chemical structure of dehydroeffusol.

A) and methanol with 0.1% formic acid (solvent B). A gradient of 65–83% B in 10 min was used, followed by 83% B for 5 min, then 83–100% B for 15–20 min and finally 100% B for 5 min. The flow rate was 4 mL min^{-1}, and detection was at 254 nm. Dehydroeffusol (DHEF) (Fig. 1) (9.6 mg, R_t 6.2 min) was obtained at a purity of >94% as determined by ^{1}H NMR analysis.

2.1.8 *Quantification of dehydroeffusol*

Analyses were performed in triplicate on an HPLC Alliance 2695 chromatographic system (Waters) equipped with a 996 PDA detector. Separations were carried out on a SunFire™ C_{18} (3.5 µm, 150 × 3.0 mm i.d.) column equipped with a guard column (10 mm × 3.0 mm i.d.). The mobile phase consisted of water with 0.1% formic acid (solvent A) and methanol with 0.1% formic acid (solvent B). The flow rate was 0.4 mL min^{-1}. A gradient of 50–100% B in 30 min was used, followed by 100% B for 5 min. Samples were dissolved in DMSO at a concentration of 500 µg mL^{-1} for the extract, and 10–50 µg mL^{-1} for dehydroeffusol. The injection volume was 10 µL, and detection was at 269 nm. A concentration of 6.0% dehydroeffusol in the extract was determined using a calibration curve: $y = 259829x + 69855$ ($r^2 = 0.9999$).

2.2 Bioassays

2.2.1 *Pathogens*

Phytophthora infestans (Mont.) de Bary was cultivated on V8 agar [200 mL L^{-1} of Campbell's V8 or Biotta® Vegetable Cocktail (Biotta AG, Tägerwilen, Switzerland), 3 g L^{-1} of $CaCO_3$, 1.5% agar, pH 6.3] at 20 °C in the dark. *Venturia inaequalis* Cooke (Wint.) was maintained on apple (*Malus domestica* Borkh.) seedlings cv. 'Jonagold' as described below. Leaves with sporulating lesions were dried at room temperature before storage in glass vessels at 4 °C in the dark. *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni was maintained on grapevine (*Vitis vinifera* L.) seedlings cv. 'Chasselas' by weekly reinoculation (described below).

2.2.2 *In vitro bioassays*

General procedures All *in vitro* experiments were performed in 96-well plates. Media appropriate for each pathogen were used, namely mineral water ('Evian') for *P. viticola*, demineralised water for *V. inaequalis* and demineralised water containing 1 mL L^{-1} of V8 medium (200 mL L^{-1} of Campbell's V8, 3 g L^{-1} of $CaCO_3$, pH 6.3) for *P. infestans*. Each test plate contained at least 16 non-treated control wells. The effect of the solvent (DMSO) alone was tested in at least eight replicates in three concentrations per experimental set.

Sporangial suspensions of *P. viticola* ($1.8–2.5 \times 10^5$ sporangia mL^{-1}) and conidial suspensions of *V. inaequalis* ($1.5–2.0 \times 10^5$ conidia mL^{-1}) were prepared by washing fresh, sporulating grapevine leaves or dried, sporulating apple leaves with demineralised water. Sporangial suspensions of *P. infestans* ($1.2–1.5 \times 10^5$ sporangia mL^{-1}) were prepared by placing mycelium dispatched from 10–14-day-old cultures into demineralised water and shaking vigorously. Suspensions were filtered over a cheesecloth, and the concentration was assessed using a Thoma cell counting chamber and adjusted to desired concentrations.

Table 1. Assessment of inhibition levels caused by plant extracts against *Phytophthora infestans*, *Venturia inaequalis* and *Plasmopara viticola* in *in vitro* experiments

Inhibition level	*P. infestans*, *V. inaequalis*	*P. viticola*
0	Similar to water control	Similar to water control
1	Distinct reduction in germination rate and/or length of germ tubes	Distinct reduction in number and/or activity of zoospores
2	No germination, or germ tubes ≤ 0.5* length of the sporangium/conidium	No zoospores germinated, or all zoospores inactive

Screening of extract library and activity profiling of microfractions To screen the library and to determine the activity of microfractions, 6 µL of the test product was added to 96-well plates containing 94 µL of the medium appropriate for each pathogen. Extracts were then serially diluted in the test plate 1:10 and 1:100 by adding 10 µL of the next higher concentration to 90 µL of the appropriate test medium, the 10 µL of the lowest concentration being discarded. Then, 20 µL of a continuously stirred pathogen suspension was added to each well, resulting in extract concentrations of 490, 49 and 4.9 µg mL^{-1}.

In a tiered approach, the whole library was screened for inhibitory activity towards *V. inaequalis* and *P. infestans*. The experiment was repeated for all extracts causing complete inhibition at 49 µg mL^{-1} or below in one or both pathogen systems, and their inhibitory effect towards *P. viticola* was evaluated as well.

Determination of minimum inhibitory concentrations (MIC_{100}) To determine the concentrations needed completely to inhibit germination of spores or activity of zoospores (MIC_{100}), *J. effusus* extract and dehydroeffusol were dissolved in DMSO at concentrations of 10 mg mL^{-1} and then serially diluted 1:1 in water down to 0.02 mg mL^{-1} (ten concentrations). A quantity of 6 µL of each test product was added to 94 µL of the appropriate medium before adding 20 µL of pathogen suspension to each well.

Assessment of inhibitory activity Effects of extracts were assessed 2–3 h (*P. viticola*), 1 day (*P. infestans*) or 2 days (*V. inaequalis*) after set-up of the experiment. All assessments were made using a binocular at magnifications of × 50–100. Inhibition levels were scored according to Table 1.

To visualise the overall inhibitory activity of microfractions, inhibition levels of the two lower concentrations were summed up, resulting in values of between 0 (no inhibition at concentrations up to 49 µg mL^{-1}) and 4 (complete inhibition down to 4.9 µg mL^{-1}).

2.2.3 *Plant-pathogen bioassays*

Plant-pathogen bioassays were carried out under semi-controlled conditions in experimental facilities (greenhouse and growth chambers). Small grapevine (cv. 'Chasselas') or apple (cv. 'Jonagold') seedlings were transplanted to individual pots (0.275 L) containing a standard substrate (Einheitserde Typ 0; Gebrüde Patzer GmbH & Co. KG, Sinntal-Jossa, Germany) previously amended with 3 g L^{-1} of a mineral fertiliser (Tardit 3 M; Hauert Günther Düngerwerke GmbH, Erlangen, Germany). Plants were grown in the greenhouse at a temperature of 18–28 °C under natural light. In wintertime, the photoperiod was extended with mercury lamps to 16 h. Plants were used for bioassays

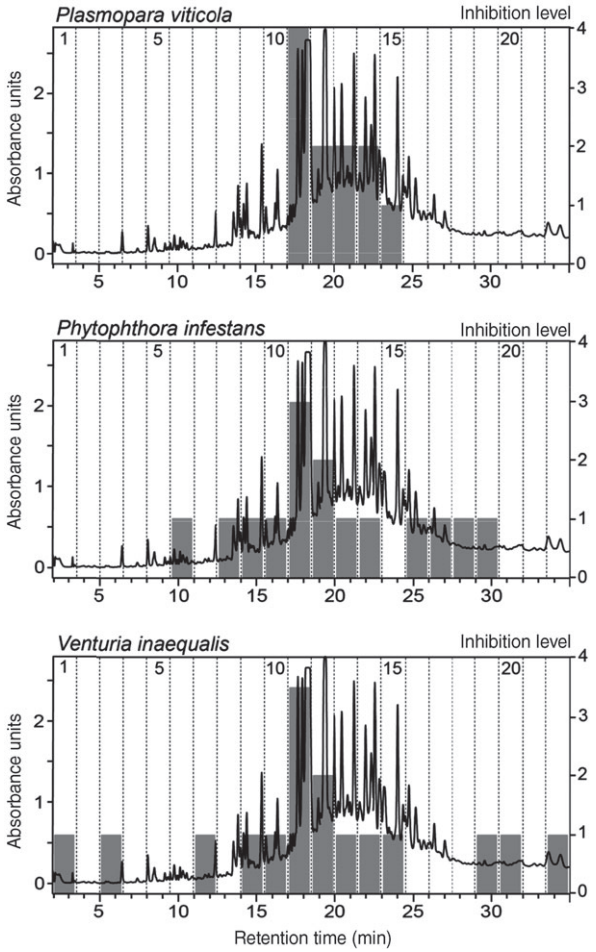

Figure 2. HPLC microfractionation of *Juncus effusus* ethyl acetate extract and *in vitro* activity of microfractions against *Plasmopara viticola*, *Phytophthora infestans* and *Venturia inaequalis*. Microfractions were collected every 90 s (22 fractions per injection). UV trace at 254 nm. Inhibition levels: level 0, $MIC_{100} > 50\ \mu g\ mL^{-1}$; level 2, complete inhibition at 50 µg mL^{-1}, MIC_{100} 5–50 µg mL^{-1}; level 3, partial inhibition at 5 µg mL^{-1}, MIC100 5-50 µg mL^{-1}; level 4, $MIC_{100} < 5\ \mu g\ mL^{-1}$.

when they had 3–4 fully developed leaves (2–3 weeks after transplanting).

Each experimental set included a non-treated non-inoculated control, a water-treated inoculated control, a standard treatment (copper hydroxide, Kocide Opti; DuPont de Nemours, Wilmington, DE) at two concentrations (300 and 30 µg mL^{-1} of copper), and at least 12 test treatments. All experiments included six replicate plants per treatment. *Juncus effusus* extract and dehydroeffusol were dissolved in DMSO at 100 mg mL^{-1} (*J. effusus* extract) or 50 mg mL^{-1} (dehydroeffusol), diluted to the highest used concentration in water and then serially diluted 1:1 in water. For *J. effusus* extract, concentrations of between 1000 and 62.5 µg mL^{-1} were tested. For dehydroeffusol, concentrations of between 60 and 1.9 µg mL^{-1} were tested, corresponding to the amount of dehydroeffusol in the *J. effusus* extract, assuming a content of 6.0% of dehydroeffusol (see Section 2.1.8). Plants were sprayed with the test products using an air-assisted hand sprayer (Compact MINI HVLP touch-up spray gun; Devilbiss, Glendale Heights, IL) until leaves (adaxial and abaxial side) were completely covered with a dense layer of small droplets. Plants were subsequently left to dry at room temperature before inoculation. *P. viticola* and *V. inaequalis* inocula were prepared from previously infected plants by washing freshly sporulating grapevine leaves or dried, infected apple leaves with water and filtering through cheesecloth. The concentration of the sporangial/conidial suspensions was adjusted to 5×10^5 sporangia mL^{-1} (*P. viticola*) or 7×10^5 conidia mL^{-1} (*V. inaequalis*) respectively. Plants were spray inoculated using an air-assisted hand sprayer on the abaxial (*P. viticola*) or the adaxial (*V. inaequalis*) leaf side. Inoculated plants were subsequently incubated at 20–21 °C and 80–99% relative humidity (RH) in the light for 24 h. Then, plants were maintained at 20 °C, 60–80% RH and a 16:8 h day/night light regime. For grapevine bioassays, 5–6 days after inoculation, plants were incubated overnight in the dark at 20 °C and 80–99% RH to promote sporulation. Disease incidence (percentage of leaves with disease symptoms) and disease severity (percentage of leaf area covered by lesions) were assessed 6–7 days after inoculation (*P. viticola*) or 10–12 days after inoculation (*V. inaequalis*). All disease assessments were made using continuous percentage values based on the European and Mediterranean Plant Protection Organisation (EPPO) standard scale.[35]

Table 2. Minimum inhibitory concentrations (MIC_{100}) of *Juncus effusus* (JE) extract and dehydroeffusol (DHEF) against *Plasmopara viticola*, *Venturia inaequalis* and *Phytophthora infestans*. The table shows means and 95% confidence limits of four independent experiments (*P. viticola*, *V. inaequalis*) or values of one experiment (*P. infestans*) respectively

	MIC_{100} JE extract (µg mL^{-1})		
Pathogen	Whole JE extract	Equivalent DHEF in extract[a]	MIC_{100} purified DHEF (µg mL^{-1})
P. viticola	24 (17; 34)[b]	1.4	4 (2; 7)
V. inaequalis	32 (19; 56)	1.9	9 (3; 23)
P. infestans	125	7.4	63

[a] Equivalent DHEF = EC_{50} of whole JE extract × 6 (percentage of DHEF in JE extract)/100.
[b] Upper and lower limit of 95% confidence interval.

2.3 Calculations

Efficacies were calculated according to Abbott[36] as

$$\left[1 - \left(A \times B^{-1}\right)\right] \times 100$$

where *A* is the disease severity on an individual plant and B is the mean disease severity of control plants.

To calculate mean and confidence intervals of MIC_{100} values, data were $\log_2$ transformed. The 95% confidence intervals were calculated from transformed data as

$$A \pm 1.96 \times B \times n^{-0.5}$$

where *A* is the mean MIC_{100}, *B* is the standard deviation MIC_{100} and *n* is the number of experiments. Data were transformed back to the linear scale.

EC_{50} values of *J. effusus* extract and of pure dehydroeffusol were calculated according to Alexander *et al.*[37] as follows:

$$EC_{50} = ConcA - \frac{(A - 50\%\ \text{maximum response}) \times (ConcA - ConcB)}{A - B}$$

where *A* and *B* are the nearest actually recorded responses on either side of the 50% maximum response ($A > 50\%$, $B < 50\%$), and

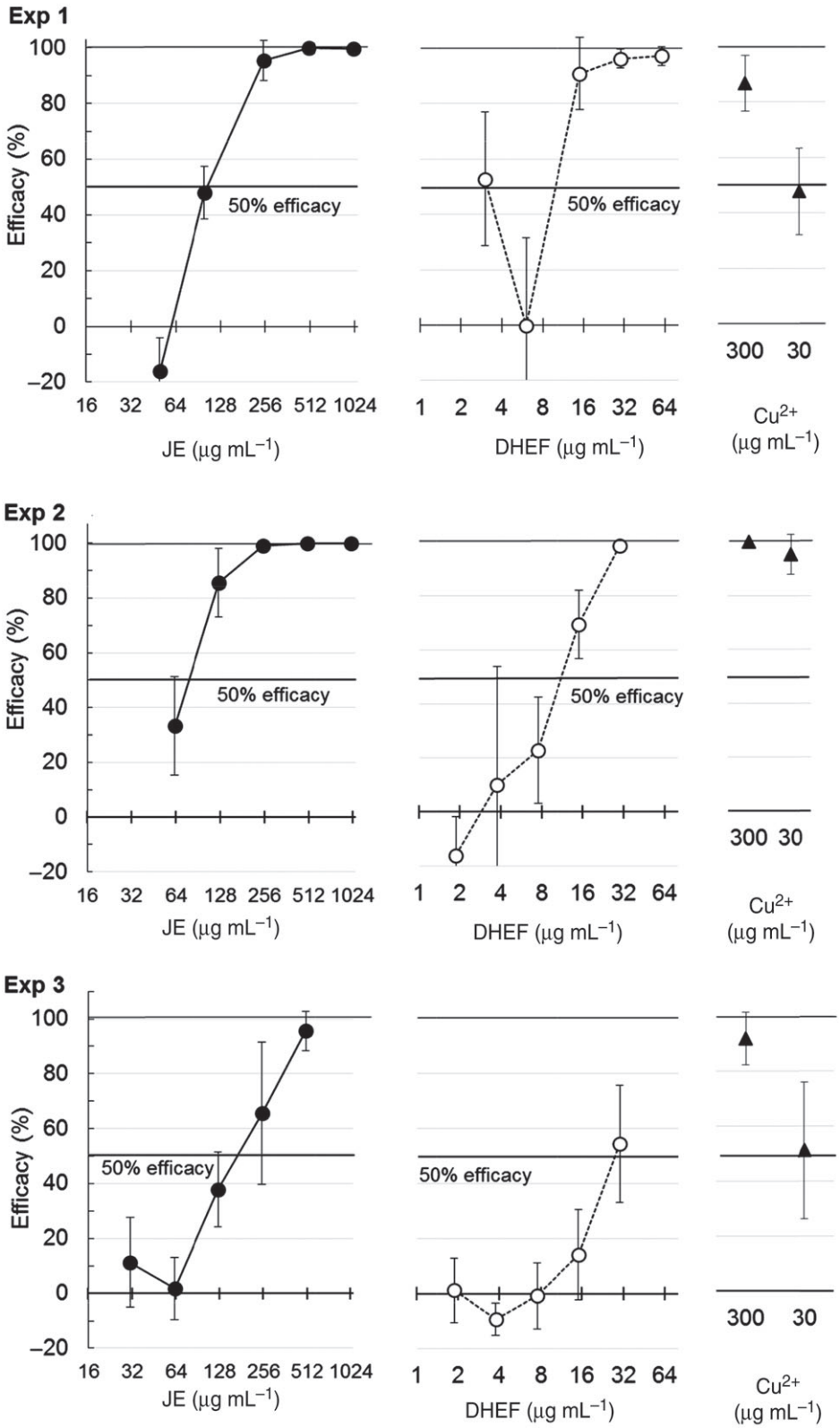

Figure 3. Dose–response curves of a *Juncus effusus* (JE) extract and dehydroeffusol (DHEF) on grapevine seedlings against *Plasmopara viticola*. The figure shows results of three independent experiments (experiments 1, 2 and 3). Each experiment included two concentrations of a copper reference (Cu^{2+}). Mean disease severities of non-treated controls were 67% (experiment 1), 75% (experiment 2) and 89% (experiment 3). The figures show means and standard deviations ($n = 6$).

ConcA and *ConcB* are the corresponding concentrations. Maximum responses were set to 100% for all experiments.

3 RESULTS

3.1 In vitro activity of a *Juncus effusus* extract and identification of its active constituent

Among approximately 120 extracts with *in vitro* fungicidal activity ($MIC_{100} \leq 50\ \mu g\ L^{-1}$) towards one or several of the investigated pathogens, the ethyl acetate extract of *J. effusus* medulla was identified as an interesting candidate owing to its remarkably low MIC_{100} values against *V. inaequalis* (32 μg mL^{-1}) and *P. viticola* (24 μg mL^{-1}) and intermediate MIC_{100} against *P. infestans* ($MIC_{100} = 125\ \mu g\ mL^{-1}$) (Table 2). Furthermore, a literature study revealed that *J. effusus* has not yet been described as a plant protection product.

The active compound(s) in the extract were identified by a process referred to as HPLC-based activity profiling.[34] During HPLC separation, microfractions were collected in a time-based manner, and subsequently tested *in vitro* for activity against *P. infestans*, *V. inaequalis* and *P. viticola*. When the bioactivity data and the chromatographic trace were compared, the activity could be mainly assigned to fraction 11 eluting between 17 and 18.5 min (Fig. 2). This fraction contained a major peak, which was isolated by a combination of preparative and semi-preparative HPLC from an extract that was prepared at larger scale. The compound was identified by mass spectrometry (*m/z* 249.7 $[M+H]^+$) and comprehensive NMR analysis[38] as the dihydrophenanthrene dehydroeffusol (Fig. 1). Dehydroeffusol was subsequently quantified by HPLC-UV analysis and found to account for 6.0% of the crude extract. The MIC_{100} values of pure dehydroeffusol *in vitro* were 4 μg mL^{-1} against *P. viticola*, 9 μg mL^{-1} against *V. inaequalis* and 63 μg mL^{-1} against *P. infestans* (Table 2).

3.2 Fungicidal activity of *Juncus effusus* extract and dehydroeffusol on grapevine and apple seedlings

Activities of *J. effusus* extract and of dehydroeffusol against downy mildew caused by *P. viticola* and against apple scab caused by *V. inaequalis* were evaluated *in planta* in dose–response experiments on susceptible seedlings under semi-controlled conditions.

3.2.1 *Grapevine – P. viticola*

The efficacy of *J. effusus* extract against downy mildew was very high in all three independent experiments, reaching 96–100% disease control in two out of three experiments at concentrations of 250 and 500 μg mL^{-1} (Fig. 3), even though disease pressure was high to very high (67–89% disease severity in control plants, data not shown). Dehydroeffusol reached efficacies of 96 and 99% disease control at a concentration of 32 μg mL^{-1} in two experiments (Fig. 3, experiments 1 and 2). In a third experiment, a maximum efficacy of 54% disease control was found for dehydroeffusol at 32 μg mL^{-1}, the highest tested concentration (Fig. 3, experiment 3). Yet, the trend of the dose–response curve indicates that higher concentrations might have resulted in higher degrees of protection. EC_{50} was between 80 and 180 μg mL^{-1} for *J. effusus* extract (Fig. 3 and Table 3) containing 5–11 μg mL^{-1} of dehydroeffusol (Table 3). EC_{50} values of purified dehydroeffusol were between 11 and 30 μg mL^{-1} (Fig. 3 and Table 3).

3.2.2 *Apple – V. inaequalis*

The efficacy of *J. effusus* extract against apple scab reached 95% disease control at 500 μg mL^{-1} (experiment 1) and 86% to 98% at 1000 μg mL^{-1} (experiments 2 and 3) (Fig. 4), even though disease pressure in the three experiments was intermediate (experiment 1, 17% diseased leaf area) to high (experiments 2 and 3, 26% to 31% diseased leaf area) (data not shown). Dehydroeffusol reached efficacies of 80% (experiment 1) and 76% disease control (experiment 2) at the highest tested concentration (32 μg mL^{-1}). The EC_{50} values were between 122 and 189 μg mL^{-1} for *J. effusus* extract (Fig. 3 and Table 3) containing 7 to 11 μg mL^{-1} of dehydroeffusol. The EC_{50} values of purified dehydroeffusol were 18 to 23 μg mL^{-1} (Table 3).

Table 3. EC_{50} of *Juncus effusus* (JE) extract and of purified dehydroeffusol (DHEF) on grapevine and apple seedlings against downy mildew caused by *Plasmopara viticola* (*Pv*) and apple scab caused by *Venturia inaequalis* (*Vi*) in three independent experiments

	EC_{50} of JE extract (μg mL^{-1})		
Bioassay	Whole JE extract	Equivalent DHEF in extract[a]	EC_{50} of purified DHEF (μg mL^{-1})
Grapevine – *Pv*			
Experiment 1	106	6	11
Experiment 2	83	5	13
Experiment 3	180	11	30
Mean	**123**	**7.3**	**18**
Appletree – *Vi*			
Experiment 1	189	11	23
Experiment 2	124	7	18
Experiment 3	122	7	19
Mean	**156**	**9**	**18**

[a] Equivalent DHEF = EC_{50} of whole JE extract × 6 (percentage of DHEF in JE extract)/100

4 DISCUSSION

The screening of a plant extract library by miniaturised *in vitro* bioassays was efficient for detecting a significant number of plant extracts with strong and reproducible direct inhibitory activity against *P. viticola*, *V. inaequalis* and/or *P. infestans* ($MIC_{100} \leq 50\ \mu g\ mL^{-1}$). A low MIC_{100} is one of several prerequisites for later profitability of a plant protection product. The ethyl acetate extract of *J. effusus* showed very strong inhibitory activity at low concentrations *in vitro* as well as *in planta*, with MIC_{100} and/or EC_{50} values much lower than those reported for other plant extracts. For example, an ethanolic plant extract of *Glycyrrhiza glabra* showed EC_{50} values of 5000 μg mL^{-1} (*J. effusus* extract in the present study 123–156 μg mL^{-1}) against bean rust on potted beans, and 10 000 μg mL^{-1} against cucumber downy mildew.[12,39] An extract from *Inula viscosa* showed ED_{90} values of between 600 and 10 000 μg mL^{-1} against downy mildew of cucumber, powdery mildew on wheat and rust on sunflower, while the ED_{90} ($ED_{90} \leq 125\ \mu g\ mL^{-1}$) against *P. viticola* on grapevine was comparable with that of the *J. effusus* extract in the present study.[14,40] Bengtsson *et al.*[16] used *Yucca schidigera* extract at a concentration of 50 000 μg mL^{-1} to obtain maximum protection of apple plants against *V. inaequalis* under controlled conditions.

So far, only little is known about the effect of *J. effusus* against plant pathogens. Inagaki *et al.*[33] reported an inhibitory effect of a leaf homogenate against *Colletotrichum lagenarium* on cucumber, but the active constituents were not characterised. In our

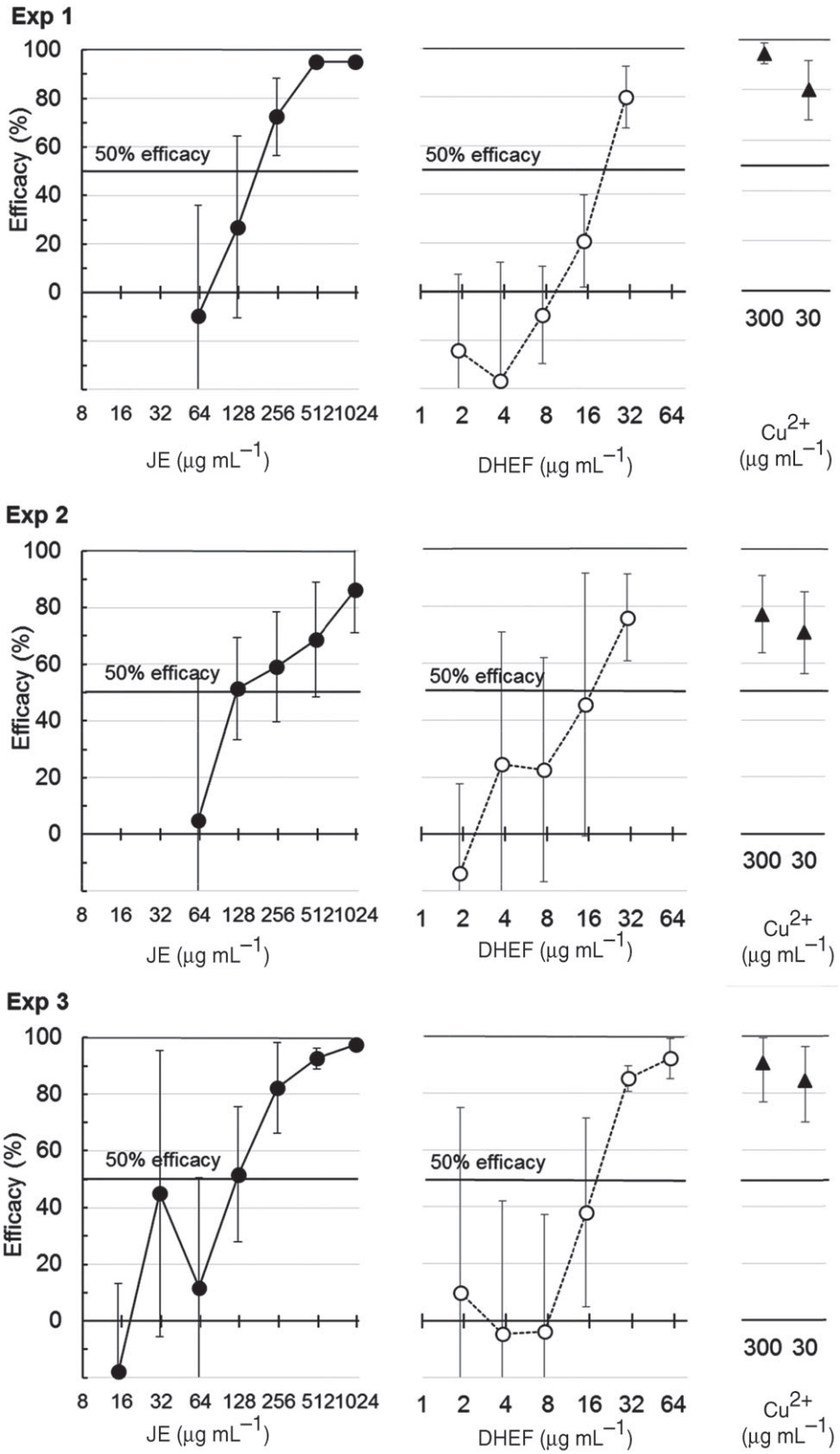

Figure 4. Dose–response curves of a *Juncus effusus* (JE) extract and dehydroeffusol (DHEF) on apple seedlings against *Venturia inaequalis*. The figure shows results of three independent experiments (experiments 1, 2 and 3). Each experiment included two concentrations of a copper reference (Cu^{2+}). Mean disease severities of non-treated controls were 17% (experiment 1), 26% (experiment 2) and 31% (experiment 3). The figures show means and standard deviations ($n = 6$).

study, dehydroeffusol was identified as the main active compound in the *J. effusus* extract, with a very low MIC_{100}/EC_{50}. Dehydroeffusol has been described before to possess antimicrobial activity against the human pathogens *Candida albicans* and *Staphylococcus aureus*.[30] Phenanthrenes have been reported from several plant families, including Orchidaceae, but the greatest number of phenanthrenes has been described from *Juncus* species, with 33 different phenanthrenes in *J. effusus* alone.[29] Phenanthrenes reportedly show cytotoxic, antimicrobial, spasmolytic, antiallergic and anti-inflammatory activities,[29] but they have not yet been exploited by the pharmaceutical or agrochemistry industry.

Dehydroeffusol is the main active compound of the *J. effusus* extract, but the facts that (i) minor activity was found in several additional microfractions and (ii) the MIC_{100} and EC_{50} of pure dehydroeffusol were higher than expected if dehydroeffusol was the only active constituent indicate that one or several other constituents might contribute to the inhibitory activity of the extract.

Besides high efficacy at low concentrations, the extract of *J. effusus* meets several other prerequisites for the development of a novel plant protection product. *J. effusus* is an almost cosmopolitan, abundant perennial species. As the medullae of *J. effusus* are used as a drug in traditional Chinese medicine, large amounts of the raw material are available on the market at relatively low prices ($US 1–60 kg^{-1}, depending on the degree of purity). Furthermore, lipophilic compounds such as dehydroeffusol are in general more rainfast than polar ones, an important prerequisite for a plant protection product used under field conditions. Nevertheless, before testing *J. effusus* extract under field conditions, a preliminary formulation with high rain fastness and good physical properties should be developed to ensure optimum performance.

In spite of the many advantages of *J. effusus* extract, some points require consideration. Firstly, some *J. effusus* phenanthrenes other than dehydroeffusol have been shown to interfere with several trophic levels in the aquatic ecosystem, as toxicity against algae (*Raphidocelis subcapitata*), a rotifer (*Brachionus calyciflorus*), a cladoceran (*Daphnia pulex*) and an anostracan (*Thamnocephalus platyurus*) have been reported.[41] Thus, ecotoxicological studies with the active ingredient dehydroeffusol and the extract should be performed at an early stage of the development of a plant protection product. Secondly, the medullae of *J. effusus* are very voluminous, with a volume of approximately 30 L kg^{-1} raw material, leading to high costs for storage/transportation. Thirdly, the low extraction yield (0.5% with the extraction method described here) needs to be improved for economic reasons, and the quality of the raw material needs attention to avoid possible contaminations, e.g. by fungicides or insecticides.

In conclusion, our results demonstrate that plants can provide potential alternatives to replace copper in organic farming, and that *J. effusus* is an interesting candidate.

ACKNOWLEDGEMENTS

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REFERENCES

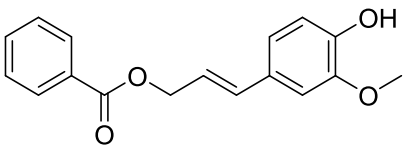
1 Bolognesi C, Genotoxicity of pesticides: a review of human biomonitoring studies. *Mut Res/Rev Mut Res* **543**(3):251–272 (2003).
2 Eijsackers H, Beneke P, Maboeta M, Louw J and Reinecke A, The implications of copper fungicide usage in vineyards for earthworm activity and resulting sustainable soil quality. *Ecotoxicol Environ Saf* **62**(1):99–111 (2005).
3 Gilliom RJ, Pesticides in US streams and groundwater. *Environ Sci Technol* **41**(10):3408–3414 (2007).
4 Mullin CA, Frazier M, Frazier JL, Ashcraft S, Simonds R, vanEngelsdorp D *et al.*, High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. *PLoS ONE* **5**(3):e9754 (2010).
5 Schwarzenbach RP, Egli T, Hofstetter TB, Von Gunten U and Wehrli B, Global water pollution and human health. *Annu Rev Environ Resour* **35**:109–136 (2010).
6 Weisenburger DD, Human health effects of agrichemical use. *Hum Pathol* **24**(6):571–576 (1993).
7 *Pflanzenschutzmittel Verzeichnis.* [Online]. Bundesamtes für Landwirtschaft, Bern, Switzerland. Available: http://www.blw.admin.ch/psm/produkte/index.html?lang=de [18 November 2015].
8 Speiser B, Tamm L, Berner A, Bickel R, Maurer V, Schneider C *et al.*, Betriebsmittelliste 2015. Research Institute of Organic Agriculture, Frick, Switzerland, 116 pp. (2015).
9 Kula C and Guske S, Auswirkungen von Kupfer auf Bodenorganismen bei langjähriger Anwendung. *Ber Biol Bundesanst* **118**:11–16 (2003).
10 Isman MB and Akhtar Y, Plant natural products as a source for developing environmentally acceptable insecticides, in *Insecticides Design Using Advanced Technologies*, ed. by Ishaaya I, Nauen R and Horowitz AR. Springer, Berlin/Heidelberg, Germany, pp. 235–248 (2007).
11 Konstantinidou-Doltsinis S and Schmitt A, Impact of treatment with plant extracts from *Reynoutria sachalinensis* (F. Schmidt) Nakai on intensity of powdery mildew severity and yield in cucumber under high disease pressure. *Crop Prot* **17**(8):649–656 (1998).
12 Scherf A, Treutwein J, Kleeberg H and Schmitt A, Efficacy of leaf extract fractions of *Glycyrrhiza glabra* L. against downy mildew of cucumber (*Pseudoperonospora cubensis*). *Eur J Plant Pathol* **134**(4):755–762 (2012).
13 Dagostin S, Formolo T, Giovannini O, Pertot I and Schmitt A, *Salvia officinalis* extract can protect grapevine against *Plasmopara viticola*. *Plant Dis* **94**(5):575–580 (2010).
14 Cohen Y, Wang W, Ben-Daniel B-H and Ben-Daniel Y, Extracts of *Inula viscosa* control downy mildew of grapes caused by *Plasmopara viticola*. *Phytopathology* **96**(4):417–424 (2006).
15 Vogt V, Cifuente D, Tonn C, Sabini L and Rosas S, Antifungal activity *in vitro* and *in vivo* of extracts and lignans isolated from *Larrea divaricata* Cav. against phytopathogenic fungus. *Ind Crops Prod* **42**:583–586 (2013).
16 Bengtsson M, Wulff E, Jørgensen HL, Pham A, Lübeck M and Hockenhull J, Comparative studies on the effects of a yucca extract and acibenzolar-*S*-methyl (ASM) on inhibition of *Venturia inaequalis* in apple leaves. *Eur J Plant Pathol* **124**(2):187–198 (2009).
17 Dagostin S, Schärer H-J, Pertot I and Tamm L, Are there alternatives to copper for controlling grapevine downy mildew in organic viticulture? *Crop Prot* **30**(7):776–788 (2011).
18 Tamm L, Speiser B and Mercier T, Proposals for regulation of botanicals, in *Regulation of Biological Control Agents*. Springer, Dordrecht, The Netherlands, pp. 289–304 (2011).
19 Potterat O and Hamburger M, Natural products in drug discovery – concepts and approaches for tracking bioactivity. *Curr Org Chem* **10**(8):899–920 (2006).
20 Su X-H, Yuan Z-P, Li C-Y, Zhong Y-J, Du H-J, Wen Y-Y *et al.*, Phenanthrenes from *Juncus effusus*. *Planta Med* **79**(15):1447–1452 (2013).
21 Xinron Y (ed.), *Traditional Chinese Medicine: a Manual from A–Z*. Springer-Verlag, Berlin/Heidelberg/New York, 665 pp. (2003).
22 Li H, Deng T, Chen Y, Feng H and Yang G, Isolation and identification of phenolic constituents from *Juncus effusus*. *Acta Pharm Sin* **42**(2):174–178 (2007).
23 Shan C, Ye Y, Jiang H and Zhang J, Study on chemical constituents isolated from *Juncus effusus*. *J Chin Med Mater* **31**(3):374–376 (2008).
24 Dong-Zhe J, Chiou GC, Iinuma M and Tanaka T, Two *p*-coumaroyl glycerides from *Juncus effusus*. *Phytochemistry* **41**(2):545–547 (1996).
25 Della Greca M, Fiorentino A, Monaco P, Previtera L and Sorrentino M, Antialgal phenylpropane glycerides from *Juncus effusus*. *Nat Prod Lett* **12**(4):263–270 (1998).
26 Della Greca M, Fiorention A, Monaco P and Previtera L, Cycloartane triterpenes from *Juncus effusus*. *Phytochemistry* **35**(4):1017–1022 (1994).
27 Corsaro MM, della Greca M, Fiorentino A, Monaco P and Previtera L, Cycloartane glucosides from *Juncus effusus*. *Phytochemistry* **37**(2):515–519 (1994).

28 Tian X-J, Li H-X, Chen Y, Wang X-Q and Yang G-Z, Studies on the chemical constituents of *Juncus effusus* L. *Lishizhen Med Mater Med Res* **9**:2121–2122 (2007).
29 Kovács A, Vasas A and Hohmann J, Natural phenanthrenes and their biological activity. *Phytochemistry* **69**(5):1084–1110 (2008).
30 Hanawa F, Okamoto M and Towers G, Antimicrobial DNA-binding photosensitizers from the common rush, *Juncus effusus*. *Photochem Photobiol* **76**(1):51–56 (2002).
31 Della Greca M, Fiorentino A, Monaco P, Pinto G, Pollio A and Previtera L, Action of antialgal compounds from *Juncus effusus* L. on *Selenastrum capricornutum*. *J Chem Ecol* **22**(3):587–603 (1996).
32 Della Greca M, Monaco P, Previtera L, Zarrelli A, Pollio A, Pinto G *et al.*, Minor bioactive dihydrophenanthrenes from *Juncus effusus*. *J Nat Prod* **60**(12):1265–1268 (1997).
33 Inagaki H, Yamaguchi A, Kato K, Kageyama C, Iyozumi H and Oki Y, Screening of weed extracts for antifungal properties against *Colletotrichum lagenarium*, the causal agent of anthracnose in cucumber. *Weed Biol Manag* **8**(4):276–283 (2008).
34 Potterat O and Hamburger M, Combined use of extract libraries and HPLC-based activity profiling for lead discovery: potential, challenges, and practical considerations. *Planta Med* **80**(14):1171–1181 (2014).
35 *Plasmopara viticola*. *EPPO Bull* **31**(2):313–317 (2001).
36 Abbott WS, A method for computing the effectiveness of an insecticide. *J Econ Entomol* **18**:265–267 (1925).
37 Alexander B, Browse DJ, Reading SJ and Benjamin IS, A simple and accurate mathematical method for calculation of the EC_{50}. *J Pharmacol Toxicol Meth* **41**(2–3):55–58 (1999).
38 Shima K, Toyota M and Asakawa Y, Phenanthrene derivatives from the medullae of *Juncus effusus*. *Phytochemistry* **30**(9):3149–3151 (1991).
39 Schuster C, Konstantinidou-Doltsinis S and Schmitt A, *Glycyrrhiza glabra* extract protects plants against important phytopathogenic fungi. *Commun Agric Appl Biol Sci* **75**(4):531 (2010).
40 Wang W, Ben-Daniel BH and Cohen Y, Control of plant diseases by extracts of *Inula viscosa*. *Phytopathology* **94**(10):1042–1047 (2004).
41 Della Greca M, Fiorentino A, Isidori M and Zarrelli A, Toxicity evaluation of natural and synthetic phenanthrenes in aquatic systems. *Environ Toxicol Chem* **20**(8):1824–1830 (2001).

3.2. PATENT: Fungicidal Compositions

Barbara Thuerig, Justine Ramseyer, Matthias Hamburger, Thomas Oberhänsli, Olivier Potterat, Hans-Jakob Schärer, and Lucius Tamm

European Patent Application, EP16175538.4



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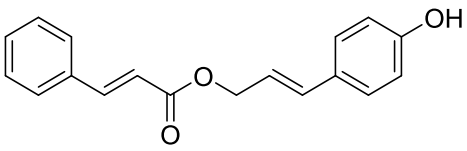


2

The petrol ether extract of Siam benzoin (SB) resin from *Styrax tonkinenis* (Styracaceae) showed promising activity against *Plasmopara viticola*, *Venturia inaequalis*, and *Phytophthora infestans*. By analogy, Sumatra benzoin (SumB) resin obtained from *Styrax benzoin* or *Styrax paralleloneurum* dissolved in ethanol was also investigated and showed potent activity against the three pathogens. On grapevine seedlings, SB and SumB in ethanol, were 100% efficient at a concentration of 1 mg/mL. The active compounds were identified as coniferyl benzoate (**1**) in SB and *p*-coumaryl cinnamate (**2**) in SumB with efficacies on grapevine seedlings ≥98% at 1 mg/mL and ≥80% at 0.25 mg/mL. The two resins and pure compounds showed also significant activities against *V. inaequalis* and *Marssonina coronaria* on apple seedlings, and against *P. infestans* on tomato seedlings. In subsequent field trials on grapevine, SB reduced significantly the infestation by downy mildew (*P. viticola*) and powdery mildew (*Uncinula necator*, anamorph *Oidium tuckeri*).

Extraction of plant material, HPLC-microfractionation, isolation of pure compounds, quantifications, writing the drafts for the phytochemical procedures in Material and Methods (1-7), and a part of Examples 1 and 4 were my contributions to this patent.

Justine Fabienne Ramseyer

FUNGICIDAL COMPOSITIONS

The present invention relates to fungicidal compositions and their applications in agriculture, and more particularly to fungicidal compositions that are particularly effective for the prevention of fungal damage and for the treatment of fungal diseases in plants and plant propagation material. Specifically, the present invention relates to fungicidal compositions comprising esters of benzoic acid and/or cinnamic acid or plant extracts comprising the same.

RELATED ART

Plant diseases have been controlled or reduced for many years by the application of pesticides including inorganic substances such copper, sulfur, potassium bicarbonate, hydrated lime or acidified clay minerals which are still frequently used (Tamm and Speiser, 2015). A number of new organic chemistry classes have in the meantime been introduced as fungicides, including dithiocarbamates, benzimidazoles, imidazoles, pyrimidines, triazoles, anilides or strobilurines (Morton and Staub, 2008).

Due to concerns related to impacts on human health and the environment, there is growing demand to replace chemical pesticides by alternatives. Moreover, under more stringent regulations, many pesticides have already been banned or are under reconsideration. Others are still allowed, but their use should be reduced or avoided wherever possible. For example, copper is widely used in conventional, integrated and organic agriculture to control devastating plant diseases such as grapevine downy mildew (*Plasmopara viticola*), potato and tomato late blight (*Phytophthora infestans*), apple scab (*Venturia inaequalis*), and a wide range of other plant pathogens, even though copper should be replaced urgently as it has an unfavorable ecotoxicological profile (Van-Zwieten et al., 2004).

Control of pathogens by means of plant-derived plant protection products can be an effective, sustainable, and environmentally friendly method for pest management in integrated pest management (IPM) and organic farming systems (Isman and Akhtar, 2007). Natural organic compounds are often easily degraded in a natural environment, e.g. by degradation by UV-light, and are thus less likely to accumulate in the environment or to cause residues on food. Extracts of selected plants, such as *Glycyrrhiza glabra* (Scherf et al., 2012), *Salvia*

officinalis (Dagostin et al., 2010) or *Larrea divaricata* (Vogt et al., 2013) have been shown to be active against plant diseases. Yet, still very few plant extracts against a limited range of diseases have been developed for commercial use and, in line, only very few are registered, for example, in Switzerland (Speiser et al., 2016). In conclusion, there is an ever growing need for plant protection products, and in particular for plant-derived plant protection products.

Siam benzoin is the resin produced by the bark of *Styrax tonkinensis* (Pierre) Craib ex Hartwich (Styracaceae), a tree growing across Thailand, Laos, Cambodia, and Vietnam. Sumatra benzoin is the resin obtained from the closely related species *Styrax benzoin* Dryand and/or *Styrax parralleloneurum* Perk (synonym *Styrax sumatranus* J J S) growing in Indonesia. Siam benzoin mainly contains benzoic acid and esters thereof, while benzoic acid derivatives are partly replaced in Sumatra benzoin by cinnamic acid in free and esterified forms such as p-coumaryl cinnamate ((Hovaneissian et al., 2008). Both balsamic resins have been traditionally used in perfumes and as incenses. More recently, cosmetic applications and medicinal properties such as positive effects on the human immune system as well as neuroprotective, neuroregenerative and anti-inflammatory properties have been described (US2004258712, WO2005/120528, WO2009/034366, US2012027868). WO1999/056547 describes herbicidal compositions and suggests the use of 3-phenyl-2-propen-1-ol benzoates as herbicides.

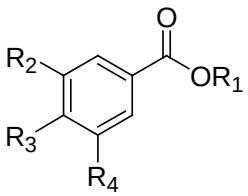
Balsam of Peru is a balsam derived from the tree *Myroxylon balsamum* (L.) Harms var. *pereirae* Royle (Fabaceae) growing in South America. Its main constituents are benzoic and cinnamic acid esters, in particular benzyl benzoate and benzyl cinnamate. Balsam of Peru has been used as a flavouring agent and in toiletries and perfumes. Balsam of Peru has been also traditionally used for the topical treatment of wounds.

SUMMARY OF THE INVENTION

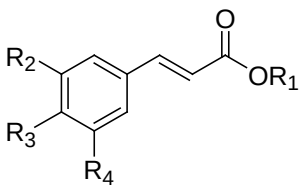
We have surprisingly found that extracts from Sumatra benzoin, Siam benzoin, and Balsam of Peru exhibit anti-fungal activity against plant pathogenic fungi. In particular, we have surprisingly found a strong inhibitory activty of Siam benzoin and Sumatra benzoin against the plant pathogens *P. viticola, V. inaequalis* and *P. infestans* and of balsam of Peru against *P. viticola* as shown by *in vitro* assays. Furthermore, we have identified as active

constituents, in particular, coniferyl benzoate, p-coumaryl cinnamate, and benzyl cinnamate. Semi-controlled bioassays with grapevine, apple and tomato seedlings confirmed furthermore efficacy of Siam benzoin and Sumatra benzoin as well as their active constituents against downy mildew caused by *P. viticola*, apple scab caused by *V. inaequalis*, Marssonina leaf drop caused by *M. coronaria* and late blight caused by *P. infestans*. In addition, the efficacy of Siam benzoin against *P. viticola* was confirmed under field conditions after appropriate formulation. Moreover, an effect against grapevine powdery mildew caused by the obligate biotroph *Oidium tuckeri* was demonstrated in the field.

Thus, in a first aspect, the present invention provides for the use of a composition as a fungicide, wherein said composition comprises at least one compound of formula (I) or formula (II)

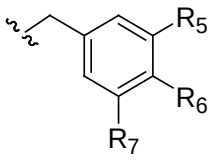


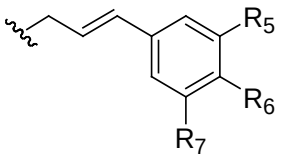
(I)



(II)

wherein

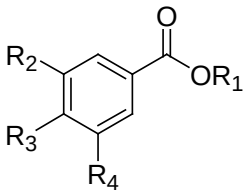
R_1 = 

or 

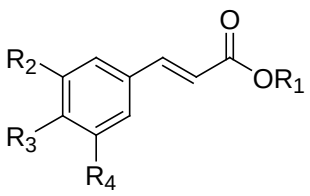
wherein

R_2, R_3, R_4, R_5, R_6 and R_7 are independently of each other H, OH or OCH_3.

In a further aspect, the present invention provides for the use as a fungicide of a compound of formula (I) or formula (II)

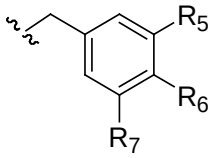
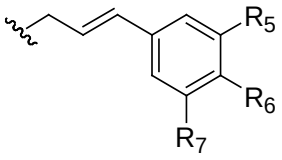


(I)



(II)

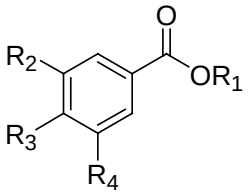
wherein

R_1 =  or 

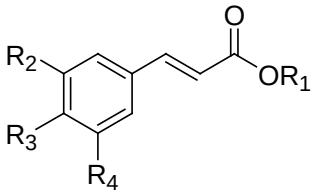
wherein

R_2, R_3, R_4, R_5, R_6 and R_7 are independently of each other H, OH or OCH_3.

In another aspect, the present invention provides for the use as a fungicide of a plant extract, wherein said plant extract comprises at least one compound of formula (I) or formula (II)

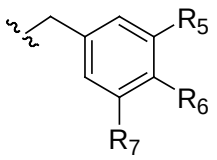
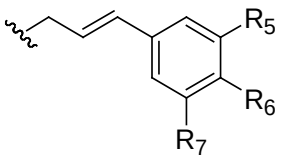


(I)



(II)

wherein

R_1 =  or 

wherein

R_2, R_3, R_4, R_5, R_6 and R_7 are independently of each other H, OH or OCH_3.

In a further aspect, the present invention provides for the use of a composition as a fungicide, wherein said composition comprises at least one plant extract, wherein said at least one plant extract is an extract from a plant of the family of *Styracaceae* or a plant of the genus *Myroxylon.* In again a further aspect, the present invention provides for the use of a composition as a fungicide, wherein said composition comprises an extract of Sumatra benzoin, Siam benzoin and Balsam of Peru. In again a further aspect, the present invention provides for the use of a composition as a fungicide, wherein said composition comprises an extract of Sumatra benzoin. In again a further aspect, the present invention provides for the use of a composition as a fungicide, wherein said composition comprises an extract of Siam benzoin. In again a further aspect, the present invention provides for the use of a composition as a fungicide, wherein said composition comprises an extract of Balsam of Peru.

In another aspect, the present invention provides for the use as a fungicide of at least

one plant extract, wherein said at least one plant extract is an extract from a plant of the family of *Styracaceae* or a plant of the genus *Myroxylon.* In another aspect, the present invention provides for the use as a fungicide of at least one plant extract, wherein said at least one plant extract is an extract from a plant of the family of *Styracaceae.* In another aspect, the present invention provides for the use as a fungicide of at least one plant extract, wherein said at least one plant extract is an extract from a plant of the genus *Myroxylon.* In a further aspect, the present invention provides for the use as a fungicide of at least one plant extract of Sumatra benzoin, Siam benzoin and Balsam of Peru. In a further aspect, the present invention provides for the use as a fungicide of at least one plant extract of Sumatra benzoin. In a further aspect, the present invention provides for the use as a fungicide of at least one plant extract of Siam benzoin. In a further aspect, the present invention provides for the use as a fungicide of at least one plant extract of Balsam of Peru.

Further aspects and embodiments of the present invention will be become apparent as this description continues.

DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs.

As used herein "a" or "an" means one or more, unless specifically indicated to mean only one.

The composition of the present invention can be used, in particular, to treat a plant, plant propagation material - such as a seed, cutting, rhizome, tuber, or bulb, for example – or soil to ameliorate or prevent damage due to infections with plant fungal pathogens.

The treatment of a plant, plant propagation material or soil with a composition of the present invention can be accomplished in several ways. The inventive composition may be applied directly to a plant seed, or to soil in which the seed is to be planted, for example, at the time of planting along with the seed. Alternatively, it may be applied to the soil after planting and germination, or to the foliage of the plant after emergence.

The term “horticultural crop” as used herein is intended to mean tree, bush and perennial vine fruits; perennial bush and tree nuts; vegetables (roots, tubers, shoots, stems, leaves, fruits and flowers of edible and mainly annual plants); aromatic and medicinal foliage,

flowers, seeds and roots (from annual or perennial plants); cut flowers, potted ornamental plants, and bedding plants (involving both annual or perennial plants); trees, shrubs, turf and ornamental grasses propagated and produced in nurseries for use in landscaping or for establishing fruit orchards or other crop production units.

The term “field crops” as used herein is intended to mean any of the herbaceous plants cultivated on a large scale in cultivated fields, primarily a grain crop, a forage crop, a sugar crop, an oil crop, a root crop or a fiber crop.

The term “fruit crop” as used herein is intended to mean a perennial, edible crop where the economic product is the fruit or is derived thereof.

When it is said that "an effective amount" of a composition according to the invention is used, it is meant that a sufficient amount of the at least one compound of formula (I) or formula (II) comprised in the inventive composition is applied to the plant, its propagation material or soil to achieve either an increase in the yield and/or the vigor of the plant, or to control a fungal infection, typically and preferably of the plant or its propagation material, preferably of the plant.

Accordingly, the expression “controlling a fungal infection” or “controlling a plant fungal pathogen” as used herein refers to invoking one or more of the following effects: (i) inhibition or arrest of fungal growth, including, reducing the rate of fungal growth or causing complete fungal growth arrest; (ii) reduction of the fungal infection incidence; (iii) reduction in fungal infection severity; and/or (iv) relief, to some extent, of one or more symptoms associated with fungal infections. By “fungal infection incidence”, typically and preferably, is meant the percentage of leaves or fruit of a given plant showing symptoms of fungal infection. Assessment is known by the skilled in the art and typically made in comparison with leaves of control and non-treated plants. By “fungal infection severity”, typically and preferably, is meant the percentage of leave, root or fruit area covered by lesions caused by said fungal disease. Assessment is known by the skilled in the art and typically made in comparison with leaves, roots or fruit of control and non-treated plants. “Symptoms associated with fungal infections” are, typically and preferably, yield losses, such as a reduced yield of tomatoes, grapes or apples, or a decrease in vigor of the plant.

The term "plant extract" as used herein is intended to mean any composition which is extracted from a plant or plant part by conventional techniques, wherein the term “plant part” comprises typically and preferably bark, wood, leaves, roots, flower buds and/or resin of said plant. Procedures and techniques of extraction and the solvents or solvent mixtures used for

said extraction are known to the skilled person in the art and are described, for example in WO2005/120528. Solvents or mixtures of solvents, typically and preferably, include water, lower alcohols such as methanol or ethanol, esters, ethers, amines, acids, polyols, alkanes or halogenated or chlorinated alkanes, and hereby protic solvents thereof such as water, alcohols, acids, primary and secondary amines and aprotic solvents thereof such as acetonitrile, DMF or DMSO. Preferred solvents for extraction are typically water, methanol, ethanol, pentane, hexane, heptane, petrol ether, acetone, chloroform, polyethylene glycol, dichloromethane, DMSO or ethyl acetate and mixtures thereof. Plants or plant parts suitable for extraction for producing a plant extract according to the invention typically and preferably have a content of at least one compound of formula (I) or formula (II), wherein said content of at least one compound of formula (I) or formula (II) is at least 1% by weight or wherein said content of the sum of all of said at least one compound of formula (I) and formula (II) is at least 1% by weight. Preferably, plants or plant parts suitable for extraction for producing a plant extract according to the invention typically and preferably have a content of at least 1% by weight of at least one compound of formula (I) or formula (II). Further preferably, plants or plant parts suitable for extraction for producing a plant extract according to the invention typically and preferably have a content of at least one compound of formula (I) or formula (II), wherein said content of at least one compound of formula (I) or formula (II) is more than 3% by weight or wherein said content of the sum of all of said at least one compound of formula (I) and formula (II) is more than 3% by weight. Again further preferably, plants or plant parts suitable for extraction have a content of more than 3% by weight of a compound of formula (I) or formula (II).

The term “resin”, as used herein and referring to the use as a fungicide of the plant extract in accordance with the present invention includes pure resins, gum-resins, oleo-gum-resins and balsams. Typically, said resin comprises at least 2% (w/w) of a compound of formula (I) or formula (II) or comprises at least 2% (w/w) by the sum of all of said at least one compound of formula (I) and formula (II), preferably said resin comprises at least 4% (w/w) of a compound of formula (I) or formula (II) or comprises at least 4% (w/w) by the sum of all of said at least one compound of formula (I) and formula (II). Typically and preferably, said resin comprises at least 2% (w/w) of compound 1, compound 2, compound 3, compound 4 or compound 5, or comprises at least 2% (w/w) by the sum of all of said compound 1, compound 2, compound 3, compound 4 or compound 5. Further preferably said resin comprises at least 4% (w/w) of compound 1, compound 2, compound 3, compound 4 or

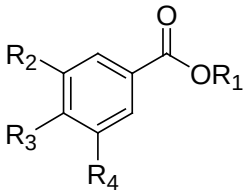
compound 5 or comprises at least 4% (w/w) by the sum of all of said compound 1, compound 2, compound 3, compound 4 and compound 5.

The term “Siam benzoin” as used herein refers to resin obtained from the bark of *Styrax tonkinensis* (Pierre) Craib ex Hartwich (Styracaceae). Siam benzoin is abbreviated herein as “SB”.

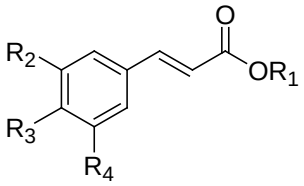
The term “Sumatra benzoin” as used herein refers to resin obtained from the bark of *Styrax benzoin* Dryand and/or *Styrax parallelloneurum* Perk (synonym *Styrax sumatranus* J J S). Sumatra benzoin is abbreviated herein as “SumB”.

The term “Balsam of Peru” as used herein refers to a balsam obtained from the bark of *Myroxylon balsamum* Harms var. *pereirae* Royle (synonyms *Myrospermum pereirae* Royle, *Myroxylon pereirae* (Royle) Klotzsch, *Toluifera pereirae* (Royle) Baill.) (Fabaceae). Balsam of Peru is abbreviated as “BP”.

Thus, in a first aspect, the present invention provides for the use of a composition as a fungicide, wherein said composition comprises at least one compound of formula (I) or formula (II)

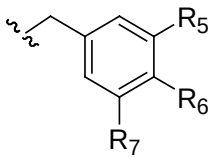
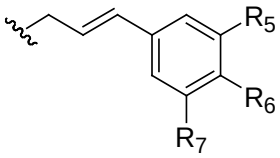


(I)



(II)

wherein

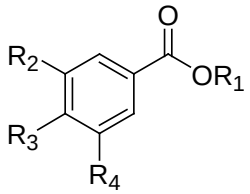
R_1 =  or 

wherein

R_2, R_3, R_4, R_5, R_6 and R_7 are independently of each other H, OH or OCH_3.

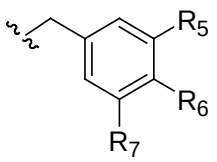
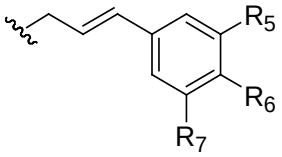
In a preferred embodiment, each of R_5, R_6 and R_7 are independently of each other H, OH or OCH_3. In a further preferred embodiment at most two of R_5, R_6 and R_7 are independently of each other OH or OCH_3. In again a further preferred embodiment R_2, R_3 and R_4 are each H. In another preferred embodiment, said R_6 is OH. In a further preferred embodiment, said R_7 is OCH_3. In again a further preferred embodiment, said R_6 is OH and said R_7 is OCH_3.

In a preferred embodiment, said composition comprises at least one compound of formula (I)



(I)

wherein

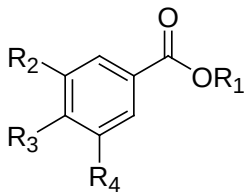
R_1 =  or 

wherein

R_2, R_3, R_4, R_5, R_6 and R_7 are independently of each other H, OH or OCH_3.

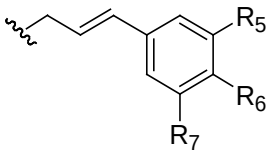
In a preferred embodiment, each of R_5, R_6 and R_7 are independently of each other H, OH or OCH_3. In a further preferred embodiment at most two of R_5, R_6 and R_7 are independently of each other OH or OCH_3. In again a further preferred embodiment R_2, R_3 and R_4 are each H. In another preferred embodiment, said R_6 is OH. In a further preferred embodiment, said R_7 is OCH_3. In again a further preferred embodiment, said R_6 is OH and said R_7 is OCH_3.

In a preferred embodiment, said composition comprises at least one compound of formula (I)



(I)

wherein

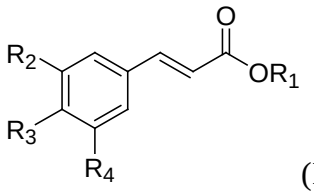
R_1 = 

wherein

R_2, R_3, R_4, R_5, R_6 and R_7 are independently of each other H, OH or OCH_3.

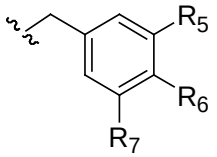
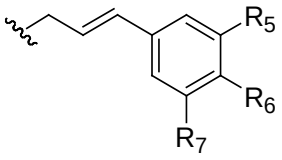
In a preferred embodiment, each of R_5, R_6 and R_7 are independently of each other H, OH or OCH_3. In a further preferred embodiment at most two of R_5, R_6 and R_7 are independently of each other OH or OCH_3. In again a further preferred embodiment R_2, R_3 and R_4 are each H. In another preferred embodiment, said R_6 is OH. In a further preferred embodiment, said R_7 is OCH_3. In again a further preferred embodiment, said R_6 is OH and said R_7 is OCH_3.

In a preferred embodiment, said composition comprises at least one compound of formula (II)



(II)

wherein

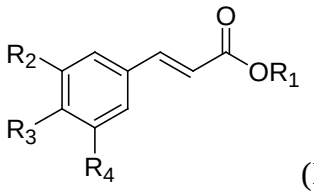
R_1 =  or 

wherein

R_2, R_3, R_4, R_5, R_6 and R_7 are independently of each other H, OH or OCH_3.

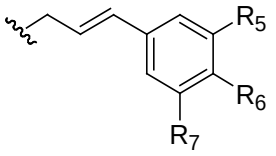
In a preferred embodiment, each of R_5, R_6 and R_7 are independently of each other H, OH or OCH_3. In a further preferred embodiment at most two of R_5, R_6 and R_7 are independently of each other OH or OCH_3. In again a further preferred embodiment R_2, R_3 and R_4 are each H. In another preferred embodiment, said R_6 is OH. In a further preferred embodiment, said R_7 is OCH_3. In again a further preferred embodiment, said R_6 is OH and said R_7 is OCH_3.

In a preferred embodiment, said composition comprises at least one compound of formula (II)



(II)

wherein

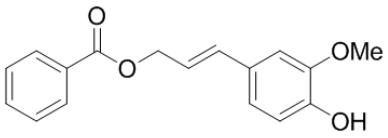
R_1 = 

wherein

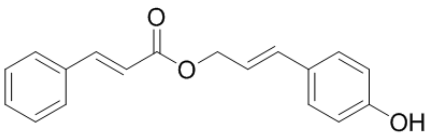
R_2, R_3, R_4, R_5, R_6 and R_7 are independently of each other H, OH or OCH_3.

In a preferred embodiment, each of R_5, R_6 and R_7 are independently of each other H, OH or OCH_3. In a further preferred embodiment at most two of R_5, R_6 and R_7 are independently of each other OH or OCH_3. In again a further preferred embodiment R_2, R_3 and R_4 are each H. In another preferred embodiment, said R_6 is OH. In a further preferred embodiment, said R_7 is OCH_3. In again a further preferred embodiment, said R_6 is OH and said R_7 is OCH_3.

In a very preferred embodiment, said at least one compound of formula (I) or formula (II) is selected from compound **1** (coniferyl benzoate), **2** (p-coumaryl cinnamate), **3** (benzyl cinnamate), **4** (benzyl benzoate) and **5** (cinnamyl cinnamate).



1



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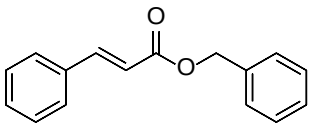
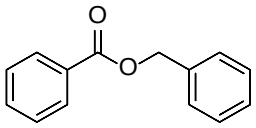


3



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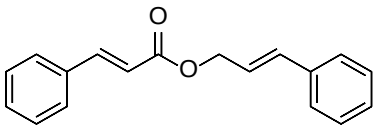
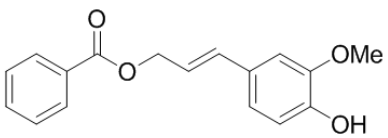
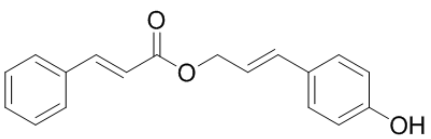


5

In a further very preferred embodiment, said at least one compound of formula (I) or formula (II) is compound **1** (coniferyl benzoate) or compound **2** (p-coumaryl cinnamate) or compound **3** (benzyl cinnamate)



1



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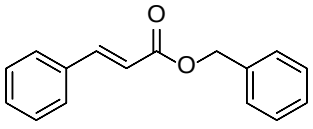


3

In another very preferred embodiment, said at least one compound of formula (I) or formula (II) is compound **1** (coniferyl benzoate). In another very preferred embodiment, said at least one compound of formula (I) or formula (II) is **2** (p-coumaryl cinnamate). In another very preferred embodiment, said at least one compound of formula (I) or formula (II) is **3** (benzyl cinnamate). In another very preferred embodiment, said at least one compound of formula (I) or formula (II) is **4** (benzyl benzoate). In another very preferred embodiment, said at least one compound of formula (I) or formula (II) is **5** (cinnamyl cinnamate).

In another very preferred embodiment, said composition comprises at least one, typically and preferably exactly one, plant extract, and wherein said plant extract comprises said at least one compound of formula (I) or formula (II). In another preferred embodiment, said at least one plant extract is an extract from a plant of the family of *Styracaceae* or a plant of the genus *Myroxylon*. In a preferred embodiment, said at least one plant extract is an extract from a plant of the genus *Styrax* or a plant of the genus *Myroxylon.*

In another very preferred embodiment, said at least one plant extract is an extract from a plant of the genus *Styrax* or a plant of the genus *Myroxylon*, and wherein said plant of the genus *Styrax* is selected from the species *Styrax tonkinensis*, *Styrax benzoin*, *Styrax paralleloneurum*, *Styrax hypoglauca* and *Styrax cascarifolia* or a subspecies or variety thereof, and wherein said plant of the genus *Myroxylon* is selected from the species *Myroxylon balsamum* and *Myroxylon peruiferum.*

In a preferred embodiment, said at least one plant extract is an extract from a plant of the genus *Styrax*, and wherein preferably said plant of the genus *Styrax* is selected from the species *Styrax tonkinensis*, *Styrax benzoin*, *Styrax paralleloneurum*, *Styrax hypoglauca* and *Styrax cascarifolia* or a subspecies or variety thereof, and wherein further preferably said plant of the genus *Styrax* is selected from the species *Styrax tonkinensis*, *Styrax benzoin* and *Styrax paralleloneurum* or a subspecies or variety thereof.

In a further preferred embodiment, said at least one plant extract is an extract from a plant of the genus *Myroxylon*, and wherein preferably said plant of the genus *Myroxylon* is selected from the species *Myroxylon balsamum* and *Myroxylon peruiferum*.

In a very preferred embodiment, said at least one plant extract is an extract from a plant of the genus *Styrax*, and wherein said plant of the genus *Styrax* is selected from the species *Styrax tonkinensis*, *Styrax benzoin*, *Styrax paralleloneurum*, *Styrax hypoglauca* and *Styrax cascarifolia* or a subspecies or variety thereof, and wherein preferably said plant of the genus

Styrax is selected from the species *Styrax tonkinensis*, *Styrax benzoin* and *Styrax paralleloneurum* or a subspecies or variety thereof.

In a further very preferred embodiment, said at least one plant extract is an extract from a plant of the genus *Myroxylon*, and wherein said plant of the genus *Myroxylon* is selected from the species *Myroxylon balsamum* and *Myroxylon peruiferum*.

In a very preferred embodiment, said at least one plant extract is an extract from a plant of the genus *Styrax*, and wherein said plant of the genus *Styrax* is selected from the species *Styrax tonkinensis*, *Styrax benzoin*, *Styrax paralleloneurum*, *Styrax hypoglauca* and *Styrax cascarifolia*, and wherein preferably said plant of the genus *Styrax* is selected from the species *Styrax tonkinensis*, *Styrax benzoin* and *Styrax paralleloneurum*.

In a further very preferred embodiment, said at least one plant extract is an extract of *Styrax tonkinensis*, *Styrax benzoin* or *Myroxylon balsamum*..

In a preferred embodiment, said at least one plant extract is an extract of bark, wood, leaves, roots, flower buds or resin of said plant. In a further very preferred embodiment, said at least one plant extract is an extract of a resin of said plant. In a further very preferred embodiment, said at least one plant extract is an extract of a resin of Siam benzoin or Sumatra benzoin or Balsam of Peru. In a further very embodiment, said at least one plant extract is an extract of a resin of Siam benzoin. In a further very embodiment, said at least one plant extract is an extract of a resin of Sumatra benzoin. In a further very embodiment, said at least one plant extract is an extract of a resin of Balsam of Peru.

In a further embodiment, said plant extract is an extract of bark, wood, leaves, roots, flower buds or resin of said plant with a protic solvent; wherein preferably said plant extract is an resin extract of said plant with a protic solvent, and further preferably wherein said protic solvent is water, methanol or ethanol, again further preferably methanol or ethanol. In a further embodiment, said plant extract is an ethanol extract from resin of said plant.

In a further embodiment, said plant extract is an extract of bark, wood, leaves, roots, flower buds or resin of said plant with an aprotic solvent; wherein preferably said plant extract is an extract of resin of said plant with a aprotic solvent, and further preferably wherein said aprotic solvent is selected from petroleum ether, hexane, heptane, acetone, ethyl acetate, DMSO, dichloromethane or chloroform, and wherein further preferably said plant extract is a petroleum ether extract from resin of said plant.

In a further embodiment, said at least one plant extract is a petroleum ether extract or an ethanol extract or a DMSO extract of bark, wood, leaves, roots, flower buds or resin of said

plant; and wherein preferably said plant extract is a petroleum ether extract or a DMSO extract or an ethanol extract of resin of said plant. In a further very preferred embodiment, said plant extract is a petroleum ether extract or an ethanol extract of a resin of said plant.

In a further embodiment, said use as a fungicide of the inventive compositions, compounds and plant extracts is for controlling a plant fungal pathogen, wherein preferably said plant fungal pathogen is selected from (i) oomycetes, (ii) ascomycetes and (iii) basidiomycetes.

Thus, the fungicidal compositions, compounds and plant extracts of the present invention are used to control plant fungal pathogens. In a preferred embodiment, said plant fungal pathogen is selected from (i) oomycetes, (ii) ascomycetes and (iii) basidiomycetes.

Typically and preferably, said (i) oomycetes are selected from the order of Peronosporales, in particular the genera *Hyaloperonospora, Peronospora, Plasmopara, Bremia, Pseudoperonospora* and *Phytophthora*; in particular the species *Hyaloperonospora brassicae* (downy mildew of several Brassicacea), *Plasmopara viticola* (grapevine downy mildew), *Plasmopara halstedii* and *Plasmopara helianthii* (sunflower downy mildew), *Pseudoperonospora cubensis* (cucurbit downy mildew) and *Pseudoperonospora humuli* (downy mildew of hops), *Bremia lactucae* (downy mildew of lettuce), *Peronospora tabacinae* (downy mildew of tobacco), *Peronospora destructor* (downy mildew of onion), *Peronospora manshurica* (downy mildew of soybean and soybean leaf spot), *Peronospora parasitica* (downy mildew of cabbage), *Peronospora farinosa* (downy mildew of chicory and beetroot), *Phytophthora phaseoli, Phytophthora citrophthora, Phytophthora capsici, Phytophthora drechsleri, Phytophthora nicotianea, Phytophthora cactorum, Phytophthora palmivora, Phytophthora cinnamoni, Phytophthora megasperma, Phytophthora parasitica, Phytophthora fragariae, Phytophthora cryptogea, Phytophthora porri, Phytophthora nicotianae, Phytophthora infestans* (downy mildew of Solanaceae, in particular late blight of potato or tomato), *Phytophthora ramorum*.

Typically and preferably, said (ii) ascomycetes are selected from the genus *Alternaria*, in particular *Alternaria solani* (early blight of Solanaceae and in particular of tomato and potato) or *Alternaria alternata*, the genus *Guignardia*, in particular *Guignardia bidwelli* (black rot of grapevine); the genus *Venturia*, in particular *Venturia inaequalis* (apple scab), *Venturia carpophila, Venturia cerasi, Venturia pyrina, Venturia pirina* (pear scabs); the genus *Oidium*, in particular powdery mildew of grapevine (*Oidium tuckerii* (synonyms *Uncinula necator, Erysiphe necator*); the genus *Erysiphe*, in particular *Erysiphe polygoni* (powdery

mildew of Cruciferae), *Erysiphe cichoracearum* (powdery mildew of cucurbits, of composites and of tomato), *Erysiphe communis* (powdery mildew of beetroot and cabbage), *Erysiphe pisi* (powdery mildew of pea and lucerne), *Erysiphe polyphaga* (powdery mildew of haricot bean and cucumber), *Erysiphe umbelliferarum* (powdery mildew of Apiaceae, in particular of carrot), *Erysiphe graminis* (synonym *Blumeria graminis*, powdery mildew of wheat and barley); the genus *Sphaeroteca*, in particular *Sphaerotheca humuli* (hop powdery mildew), *Sphaerotheca fuligena*; the genus *Leveillula*, in particular *Leveillula taurica* (onion powdery mildew), the genus *Podosphaera*, in particular *Podosphaera leucotricha* (apple powdery mildew); the genus *Marssonina*, in particular *Marssonina coronaria* (synonym *Diplocarpon mali*); the genus *Taphrina*, in particular *Taphrina deformans* (peach leaf curl); the genus *Septoria*, in particular *Septoria nodorum* or *Septoria tritici* (Septoria disease of cereals), the genus *Sclerotinia*, in particular *Sclerotinia sclerotinium*; the genus *Pseudocercosporella*, in particular *Pseudocercosporella herpotrichoides* (eyespot of cereals); the genus *Botrytis*, in particular *Botrytis cinerea* (grapevine, vegetable and market garden crops, pea and the like); the genus *Phomopsis*, in particular *Phomopsis viticola* (excoriosis of grapevine); the genus *Pyrenospora*; the genus *Helminthosporium*, in particular *Helminthosporium tritici repentis* (yellow leaf spot of wheat) or *Helminthosporium teres* (yellow leaf spot of barley); or the genera *Drechslera* or *Pyrenophora*.

Typically and preferably, said (iii) basidiomycetes are selected from the genus *Puccinia*, in particular *Puccinia recondita* or *Puccinia striiformis* (wheat rust), *Puccinia triticina, Puccinia hordei*; the genus *Phacopsora*, in particular *Phacopsora pachyrhizi*; or the genus *Rhizoctonia*, in particular *Rhizoctonia solani*.

In a further preferred embodiment, said plant fungal pathogen is selected from (i) oomycetes, (ii) ascomycetes and (iii) basidiomycetes, and wherein said (i) oomycetes are selected from the genera *Hyaloperonospora, Peronospora, Plasmopara, Bremia, Pseudoperonospora* and *Phytophthora*; and wherein said (ii) ascomycetes are selected from the genera *Alternaria, Guignardia, Venturia, Oidium, Erysiphe, Sphaeroteca, Leveillula, Podosphaera, Marssonina, Taphrina, Septoria, Sclerotinia, Pseudocercosporella, Botrytis, Phomopsis, Pyrenospora*; *Helminthosporium, Drechslera* and *Pyrenophora*; and wherein said (iii) basidiomycetes are selected from the genera *Puccinia, Phacopsora*, and *Rhizoctonia*.

In a further very preferred embodiment, said plant fungal pathogen is selected from *P. viticola, V. inaequalis, P. infestans, M. coronaria* and *Oidium tuckeri*.

In a further embodiment, said use as a fungicide of the inventive compositions,

compounds and plant extracts is for controlling a fungal infection of a plant, plant propagation material or soil, preferably of a plant or plant propagation material, and again further preferably of a plant. In a further embodiment, said fungal infection is a fungal infection of a crop or a forestry plant. In a further very embodiment, said fungal infection is a fungal infection of a crop. In a further very embodiment, said fungal infection is a fungal infection of a crop selected from a horticultural crop or a field crop. In a further very embodiment, said fungal infection is a fungal infection of a crop selected from a horticultural crop preferably of a fruit crop or a vegetable. In a further very embodiment, said fungal infection is a fungal infection of a horticultural crop. In a further very embodiment, said fungal infection is a fungal infection of a field crop. In a further very embodiment, said fungal infection is a fungal infection of a fruit crop. In a further very embodiment, said fungal infection is a fungal infection of a vegetable. In a further very preferred embodiment, said fungal infection is a fungal infection of a fruit crop selected from a grapevine plant or an apple tree. In a further very preferred embodiment, said fungal infection is a fungal infection of a vegetable, wherein said vegetable is a tomato plant.

In a further very preferred embodiment, said fungal infection is a fungal infection of a fruit crop or of a vegetable, wherein said fruit crop is a grapevine plant or an apple tree and said vegetable is a tomato plant. In a further very preferred embodiment, said fungal infection is a fungal infection of grapevine plant, apple trees or tomato plants.

In a further very preferred embodiment, said fungal infection is a fungal infection of a crop selected from a fruit crop or a vegetable, wherein preferably said fruit crop is a grapevine plant or an apple tree and wherein preferably said vegetable is a tomato plant.

In a very further preferred embodiment, the composition in accordance with the present invention is used for controlling a plant fungal pathogen and for controlling a fungal infection selected from (i) a fungal infection of grapevine with *P. viticola* (grapevine downy) and/or *Oidium tuckeri* (powdery mildew); (ii) a fungal infection of apple trees with *V. inaequalis* (apple scab) and/or *Diplocarpon mali/Marssonina coronaria* (Marssonina leaf drop); or (iii) a fungal infection of tomato plants with *P. infestans* (tomato late blight).

A further preferred embodiment includes the instance where the plant infected with said plant fungal pathogen is selected from a grapevine plant, an apple tree and a tomato plant.

In a further embodiment, said composition further comprises an agriculturally acceptable excipient. Typically and preferably the inventive composition comprises one or more agriculturally acceptable excipients. The term “agriculturally acceptable excipient” as

used herein refers to an excipient that is not unacceptably damaging to a plant or its environment, and/or not unsafe to the user or others that may be exposed to the material when used as described herein. In a preferred embodiment, said agriculturally acceptable excipient may comprise a liquid or solid carrier, surface-active agents, crystallisation inhibitors, viscosity-modifying substances, dyes, anti-oxidants, foaming agents, light absorbers, mixing aids, antifoams, complexing agents, neutralising or pH-modifying substances and buffers, thickeners, anti-freezes, microbiocides, stabilizers, and also liquid and solid fertilisers. The compositions according to the invention can additionally include an efficacy-enhancing additive commonly referred to as an adjuvant.

In a further very preferred embodiment, said controlling said plant fungal pathogen or said controlling said fungal infection of said plant, plant propagation material or soil, comprises applying an effective amount of said composition to said plant, plant propagation material or soil, preferably to said plant or plant propagation material, and further preferably to said plant, wherein preferably said effective amount of said composition applied to said plant, plant propagation material or soil, preferably to said plant or plant propagation material, and further preferably to said plant, is an amount of said composition sufficient to provide a concentration of said at least one compound of formula (I) or formula (II) of 0.02% or a concentration of 0.02%, of the sum of all of said at least one compound of formula (I) and formula (II), or to provide a concentration of said at least one plant extract, preferably said extract of a resin of said plant, of 0.05%.

In a further preferred embodiment, said plant extract is an extract of a resin of said plant, and wherein the concentration of said resin in said extract is from 0.1 g l^{-1} to 100 g l^{-1} , preferably wherein the concentration of said resin in said extract is from 1 g l^{-1} to 10 g l^{-1} .

The compounds, plant extracts and compositions according to the invention can be used as fungicides in unmodified form. Typically and preferably, said compounds, plant extracts and said compositions according to the invention will be further converted to formulations, such as wettable powders, water-dispersible granules, emulsifiable granules, emulsifiable concentrates, microemulsion concentrates, oil-in-water (EW) or water-in-oil (WO) emulsions, suspoemulsions, capsule suspensions or other formulations as, for example, defined in the Manual on Development and Use of FAO Specifications for Plant Protection Products, March 2006 revision of the First edition. Such formulations can either be used directly or are diluted prior to use. Dilution media for the formulations can be, for example, water, liquid fertilisers, oils or solvents. Water is generally the preferred carrier for the dilution of the formulations.

The formulations can be applied as such or in diluted form through suitable ground spray equipment or through aerial application known to the person skilled in the art.

Thus, in a further very preferred embodiment, said composition is adapted as a formulation, wherein preferably said formulation is selected from a wettable powder, an emulsifiable concentrate, a water-dispersible granule, an emulsifiable granule, a microemulsion concentrate, an oil-in-water (EW) or water-in-oil (WO) emulsion, a suspo-emulsion and a capsule suspension. In a further very preferred embodiment, said composition is adapted as a formulation, wherein said formulation is selected from a wettable powder, an emulsifiable concentrate, a water-dispersible granule or an emulsifiable granule.

The formulations are produced in a manner known for the person skilled in the art, for example by mixing the compounds, plant extracts and compositions according to the invention with formulation adjuvants in order to obtain compositions in the form of finely divided solids, granules, solutions, dispersions or emulsions. The compounds, plant extracts and compositions according to the invention can also be contained in fine microcapsules consisting of a core and a polymeric shell. The formulation adjuvants suitable for the preparation of the compositions according to the invention are known to those skilled in the art.

Liquid carriers which may be used are, for example, water or organic solvents. Organic solvents comprise aromatic solvents such as toluene, xylene, aromatic hydrocarbon blends with boiling ranges between 150 and 300 °C known under various trademarks like Aromatic®, Solvesso®, Shellsol A®, Caromax®, Hydrosol®; paraffinic and isoparaffinic hydrocarbon solvents with boiling ranges between 150 and 360 °C known for example under the trademarks Exxsol®, Varsol®, Isopar® or Shellsol T®; hydrocarbons such as cyclohexane, tetrahydronaphthalene (tetralin), decahydronaphthalene, alpha-pinene, d-limonene; ester solvents such as ethyl acetate, n/iso-butyl acetate, amyl acetate, isobornyl acetate; alkyl esters of lactic acid; alkyl and aryl esters of benzoic acid such as methyl benzoate, benzyl benzoate, dipropyleneglycol dibenzoate; polar solvents like N-methyl pyrrolidone, N-ethyl pyrrolidone, C_3-C_{18}-alkyl pyrrolidones, dimethylsulfoxide, gamma-butyrolactone, N,N-dimethylformamide, N,N-dimethylacetamide, N,N-dimethyllactamide, C_4–C_{18} fatty acid dimethylamides, benzoic acid dimethylamide, methyl-isobutyl ketone, isoamyl ketone, 2-heptanone, cyclohexanone, isophorone, mesityl oxide, acetophenone, ethylene carbonate, propylene carbonate, butylene carbonate; alcoholic solvents and diluents such as methanol, ethanol, propanol, n/iso-butanol, n/iso-pentanol, 2-ethyl hexanol, n-octanol,

tetrahydrofurfuryl alkohol, 2-methyl-2,4-pentanediol, 4-hydroxy-4-methyl-2-pentanon, cyclohexanol, benzyl alcohol, ethylene glycol, ethylene glycol butyl ether, ethylene glycol methyl ether, diethylene glycol, diethylene glycol butyl ether, diethylene glycol ethyl ether, diethylene glycol methyl ether, propylene glycol, dipropylene glycol, dipropylene glycol methyl ether and other similar glycol ether solvents, polyethylene glycol (PEG 400), glycerol, glycerol acetate, glycerol diacetate, glycerol triacetate; fatty acid esters such as methyl octanoate, isopropyl myristate, methyl laurate, methyl oleate, mixture of C8-C10 fatty acid methyl esters, rape seed oil methyl and ethyl esters, soy bean oil methyl and ethyl esters, vegetable oils; fatty acids such as oleic acid, linoleic acid, linolenic acid; phosphate and phosphonate esters such as triethyl phosphate, C3-C18-alkyl phosphates, alkyl-aryl phosphates, esters of alkylphosphonic acid.

Suitable solid carriers are, for example, ground natural minerals such as kaolins, clays, attapulgite clay, precipitated or fumed silica, talc, titanium dioxide diatomaceous earth, limestone, calcium carbonate, bentonite or ground organic materials such as sawdust, coconut shells, maize cobs, cottonseed husks, wheatmeal, soybean flour, ground walnut shells, lignin and similar materials.

A large number of surface-active substances can be used both in solid and in liquid formulations. Surface-active substances may be anionic, cationic, amphoteric, non-ionic or polymeric and they may be used as emulsifiying, wetting, dispersing or suspending agents or for other purposes. Typical surface-active substances include, for example, salts of alkyl sulfates, such as sodium lauryl sulphate; salts of alkylaryl sulfonates, such as calcium or sodium dodecylbenzene sulfonate; salts of alkylnaphthalene sulfonates, such as sodium dibutylnaphthalene sulfonate; dialkyl esters of sulfosuccinate salts, such as sodium di(2-ethylhexyl)sulfosuccinate; sodium salts of naphthalene sulfonic acid; formaldehyde condensation products; calcium or sodium ligninsulfonates; ethoxylated castor oils with 10 – 40 mol% ethylene oxide; alkylphenol-alkylene oxide addition products, such as nonylphenol ethoxylates; alcohol-alkylene oxide addition products, such as tridecyl alcohol ethoxylates; soaps, such as sodium stearate; sorbitol esters, such as sorbitol oleate; polyethylene glycol esters of fatty acids, such as polyethylene glycol stearate; block copolymers of ethylene oxide and propylene oxide; and salts of mono- and di-alkyl phosphate esters; and also further substances described e.g. in "McCutcheon's Detergents and Emulsifiers Annual", MC Publishing Corp., Ridgewood, New Jersey, 1981.

Further adjuvants which can be used in the compositions of the invention include

crystallisation inhibitors, viscosity-modifying substances, dyes, anti-oxidants, foaming agents, light absorbers, mixing aids, antifoams, complexing agents, neutralising or pH-modifying substances and buffers, thickeners, anti-freezes, microbiocides, stabilizers, and also liquid and solid fertilisers. The compositions according to the invention can additionally include an efficacy-enhancing additive commonly referred to as an adjuvant. Examples of such adjuvants are oils of vegetable or animal origin, alkyl esters of such oils or mixtures of such oils and oil derivatives, or mineral oils. Especially preferred oil additives comprise alkyl esters of C_8-C_{22} fatty acids, especially the methyl derivatives of C_{12}-C_{18} fatty acids, for example the methyl esters of lauric acid, palmitic acid and oleic acid and mixtures thereof. The application and action of the oil additives can be further improved by combining them with surface-active substances, such as non-ionic, anionic, cationic or amphoteric surfactants. A preferred anionic surfactant is the calcium salt of dodecylbenzene sulfonic acid. Preferred non-ionic surfactants are ethoxylates of fatty alcohols. Special preference is given to ethoxylated C_{12}-C_{22} fatty alcohols having a degree of ethoxylation of from 5 to 40%. Also preferred are silicone surfactants, especially polyalkyl-oxide-modified heptamethyltrisiloxanes, which are commercially available e.g. as Silwet L-77®. The concentration of surface-active substances in relation to the total additive is generally from 1 to 30% by weight. The said surface-active substances may also be used as efficacy enhancing additives alone without oil. The oil additive can be added to the spray tank in the desired concentration after the spray mixture has been prepared or built-in into the formulation.

The fungicidal compositions according to the invention generally comprise between 0.1 and 95% by weight of fungicidal compounds or plant extracts and preferably between 0.5 and 90%, more preferably from 5 to 99.9 % by weight of a formulation adjuvant.

In a further aspect, the present invention provides for the use of a composition as a fungicide, wherein said composition comprises at least one plant extract, wherein said at least one plant extract is an extract from a plant of the family of *Styracaceae* or a plant of the genus *Myroxylon.* In a preferred embodiment of said use, said at least one plant extract is an extract from the genus *Styrax* or a plant of the genus *Myroxylon.* In a very preferred embodiment, said at least one plant extract is an extract of *Styrax tonkinensis*, *Styrax benzoin*, *Styrax paralleloneurum* or *Myroxylon balsamum*, and wherein preferably said at least one plant extract is an extract of a resin of Siam benzoin or Sumatra benzoin or Balsam of Peru. In a further preferred embodiment, said at least one plant extract is an extract from a plant of the genus *Styrax*, and wherein preferably said plant of the genus *Styrax* is selected from the

species *Styrax tonkinensis*, *Styrax benzoin*, *Styrax paralleloneurum*, *Styrax hypoglauca* and *Styrax cascarifolia* or a subspecies or variety thereof, and wherein further preferably said plant of the genus *Styrax* is selected from the species *Styrax tonkinensis*, *Styrax benzoin* and *Styrax paralleloneurum* or a subspecies or variety thereof. In a further preferred embodiment, said at least one plant extract is an extract from a plant of the genus *Styrax*, and wherein said plant of the genus *Styrax* is from the species *Styrax tonkinensis* or a subspecies or variety thereof, and wherein further preferably said plant of the genus *Styrax* is selected the species *Styrax tonkinensis*. In a further preferred embodiment, said at least one plant extract is an extract from a plant of the genus *Styrax*, and wherein said plant of the genus *Styrax* is selected from the species *Styrax benzoin*, *Styrax paralleloneurum* or a subspecies or variety thereof, and wherein further preferably said plant of the genus *Styrax* is selected from the species *Styrax benzoin* and *Styrax paralleloneurum*. In a further preferred embodiment, said at least one plant extract is an extract from a plant of the genus *Myroxylon*, and wherein preferably said plant of the genus *Myroxylon* is selected from the species *Myroxylon balsamum* and *Myroxylon peruiferum*. In again a further preferred embodiment, wherein said plant of the genus *Styrax* is selected from the species *Styrax tonkinensis*, *Styrax benzoin*, *Styrax paralleloneurum*, *Styrax hypoglauca* and *Styrax cascarifolia* or a subspecies or variety thereof, and wherein said plant of the genus *Myroxylon* is selected from the species *Myroxylon balsamum* and *Myroxylon peruiferum*. In again a further preferred embodiment, said at least one plant extract is an extract of *Styrax tonkinensis*, *Styrax benzoin*, *Styrax paralleloneurum* or *Myroxylon balsamum*, and wherein preferably said at least one plant extract is an extract of a resin of Siam benzoin or Sumatra benzoin or Balsam of Peru. In a very preferred embodiment, said composition comprises at least one plant extract, wherein said at least one plant extract is an extract of *Styrax tonkinensis*, *Styrax benzoin*, *Styrax paralleloneurum* or *Myroxylon balsamum*, and wherein preferably said at least one plant extract is an extract of a resin of Siam benzoin or Sumatra benzoin or Balsam of Peru. In a very preferred embodiment, said composition comprises at least one plant extract, wherein said at least one plant extract is an extract of *Styrax tonkinensis*, and wherein said at least one plant extract is an extract of a resin of Siam benzoin. In a very preferred embodiment, said composition comprises at least one plant extract, wherein said at least one plant extract is an extract of *Styrax benzoin* or *Styrax paralleloneurum*, and wherein said at least one plant extract is an extract of a resin of Sumatra benzoin. In a very preferred embodiment, said composition comprises at least one plant extract, wherein said at least one plant extract is an

extract of *Myroxylon balsamum*, and wherein said at least one plant extract is an extract of a resin of Balsam of Peru.

In again a further aspect, the present invention provides for the use of a composition as a fungicide, wherein said composition comprises an extract of Sumatra benzoin, Siam benzoin and Balsam of Peru. In again a further aspect, the present invention provides for the use of a composition as a fungicide, wherein said composition comprises an extract of Sumatra benzoin. In again a further aspect, the present invention provides for the use of a composition as a fungicide, wherein said composition comprises an extract of Sumatra benzoin. In again a further aspect, the present invention provides for the use of a composition as a fungicide, wherein said composition comprises an extract of Balsam of Peru.

EXAMPLES

Various aspects of the invention make use of the following materials and methods and are illustrated by the following non-limiting examples.

MATERIALS AND METHODS

Phytochemistry

1. Chemicals: Solvents and formic acid were obtained from Scharlau (Barcelona, Spain). For extraction, technical grade solvents were used after redistillation. For high-performance liquid chromatography (HPLC), HPLC-grade solvents were used. HPLC grade water was obtained from a MilliQ water purification system (Merck Millipore, Darmstadt, Germany). Deuterated solvents for NMR analysis were purchased from ARMAR Chemicals (Döttingen, Switzerland).

2. Plant Material: Siam benzoin, further referred to as “SB” and Sumatra benzoin (below referred to as “SumB”) were purchased from Alfred Galke GmbH (Gittelde, Germany). The plant material was imported from Laos (SB) or Java, Indonesia (SumB), respectively. Balsam of Peru (below referred to as “BP”) was purchased from Hänseler AG (Herisau, Switzerland). Voucher specimens (Nr. 900, SB; Nr. 959, SumB, Nr. 988, BP) are kept at the Division of Pharmaceutical Biology, University of Basel, Switzerland.

3. General Procedures: Preparative HPLC of Siam benzoin was performed on a LC8A Preparative Liquid Chromatograph consisting of a SCL-10VP controller, LC-8A binary pumps, and a UV-vis SPD-M10A detector (Shimadzu, Kyoto, Japan), using a SunFire™ Prep C_{18} OBD column (5 µm, 150 x 30 mm i.d., Waters, Milford, MA, USA). For Sumatra benzoin and balsam of Peru, preparative HPLC was carried out on a puriFlash® 4100-250 system (Interchim, Montluçon, France) equipped with a SunFire™ Prep C_{18} OBD column (5 µm, 150 x 30 mm i.d., Waters, Milford, MA, USA). Semi-preparative HPLC was performed on an Agilent 1100 Series with a PDA detector (Santa Clara, CA, USA) connected to a FC204 fraction collector (Gilson, Middleton, WI, USA). Separations were carried out on a SunFire™ Prep C_{18} column (5 µm, 150 x 10 mm i.d., Waters) equipped with a guard column (10 x 10 mm i.d.). NMR spectra were recorded on a 500 MHz Avance III™ spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a 1 mm TXI microprobe. Standard pulse sequences of the software package Topspin 3.0 were used.

4. Extraction: For the preparation of a petroleum ether extract of Siam benzoin the resin was frozen with liquid nitrogen and milled with an Universal Mill M20 (IKA®-Werke, Staufen im Breisgau, Germany). The powdered material (9 kg) was separated in two portions of about 4.5 kg, then mixed with sea sand and rough sand (1:2). Each portion was then filled in a column and percolated during 4 days with about 45 L of petroleum ether. After evaporation under reduced pressure, 340 g of extract were obtained (yield: 3.8 %). The petroleum ether extract is referred herein as “SB-PE” or “SumB-PE”. Alternatively, Siam benzoin and Sumatra benzoin were dissolved directly in ethanol (250 g l^{-1}). Both resins are fully soluble in EtOH with exception of some pieces of bark trapped in the resin. Balsam of Peru was dissolved in ethanol (250 gl^{-1}). These ethanol extracts are referred herein as “SB-EtOH”, “SumB-EtOH”, or “BP-EtOH”.

5. HPLC Microfractionation: Microfractionation was performed by semi-preparative HPLC. The mobile phase consisted of water with 0.1 % formic acid (Solvent A) and acetonitrile containing 0.1 % formic acid (Solvent B). A gradient of 5 to 100 % B in 30 min was used, followed by isocratic conditions of 100 % B for 5 min. The flow rate was 4.0 ml min^{-1}. The extract was dissolved in DMSO at a concentration of 50 mg ml^{-1}, centrifuged, and filtered. Two injections of 200 µL were performed (20 mg of extract in total). Microfractions were collected every 90 sec from 1 to 34 min (22 fractions per injection). After removal of the eluent in a Genevac EZ-2 evaporator (Stone Ridge, NY, USA), the fractions were re-dissolved in 300 µL of methanol. The corresponding fractions obtained from the two

separations were combined and re-dried. Before testing in *in vitro* bioassays, the fractions were re-dissolved in 70 µL DMSO.

6. Isolation of the Active Constituents:

6.1. Coniferyl benzoate: A portion (600 mg) of Siam benzoin petroleum ether extract was separated by preparative HPLC in 6 aliquots dissolved in DMSO to afford pure coniferyl benzoate (192 mg, t_R = 10.7 min). The sample was dissolved immediately prior each injection. The mobile phase consisted of water with 0.1 % formic acid (Solvent A) and acetonitrile with 0.1 % formic acid (Solvent B). Isocratic elution with 60 % B was used. The flow rate was 20 ml min^{-1}. Purity of coniferyl benzoate was ≥98% as determined by ^{1}HNMR analysis.

6.2. Coumaryl cinnamate: Separation of Sumatra benzoin (6 x 100 mg dissolved in DMSO) by preparative HPLC afforded pure coumaryl cinnamate (135 mg, t_R = 16.5 min). The mobile phase consisted of water with 0.1 % formic acid (Solvent A) and acetonitrile with 0.1 % formic acid (Solvent B). Isocratic elution with 60 % B was used. The flow rate was 20 ml min^{-1}. Purity of coumaryl cinnamate was ≥98% as determined by ^{1}HNMR analysis.

6.3 *Benzyl cinnamate*: Separation of Balsam of Peru (270 mg) by preparative HPLC in 4 aliquots provided pure benzyl cinnamate (t_R = 19.3 min, 55 mg). The mobile phase consisted of water (solvent A) and acetonitrile (solvent B). Isocratic elution with 50% B for 2 min, followed by a gradient of 50 to 100 % B in 20 min was applied. The flow rate was 20 ml min^{-1}. Purity was ≥95% as determined by ^{1}HNMR analysis.

7. Quantification of the Active Constituents:

7.1.: Coniferyl benzoate: Analyses were performed in triplicate on a HPLC Alliance 2695 chromatographic system (Waters) equipped with a 996 PDA detector. Separation were carried out on a SunFire™ C_{18} (3.5 µm, 150 x 3.0 mm i.d., Waters) column equipped with a guard column (10 mm x 3.0 mm i.d.). The mobile phase consisted of water with 0.1 % formic acid (Solvent A) and acetonitrile with 0.1 % formic acid (Solvent B). The flow rate was 0.4 ml min^{-1}. A gradient of 50 to 100 % B in 30 min, followed by isocratic conditions of 100 % B for 5 min was used. Samples were dissolved in acetonitrile at a concentration of 100 µg ml^{-1} for the extract and 25 - 125 µg ml^{-1} for coniferyl benzoate. The autosampler temperature was set at 4°C. The injection volume was 10 µl. Detection was at 267 nm. A calibration curve was used to determine the concentration of coniferyl benzoate in the extract: 84047x + 399888 (r^2 = 0.9965).

7.2.: Coumaryl cinnamate: Analyses were performed in triplicate on a Binary Gradient

prominence LCMS/MS 8030 system (Shimadzu) equipped with a PDA detector. Separation were carried out on a SunFire^{TM} C_{18} (3.5 µm, 150 x 3.0 mm i.d.) column equipped with a guard column (10 mm x 3.0 mm i.d.). The mobile phase consisted of water with 0.1 % formic acid (Solvent A) and acetonitrile with 0.1 % formic acid (Solvent B). The flow rate was 0.4 ml min^{-1}. A gradient of 30 to 70 % B in 30 min, followed by isocratic conditions of 100 % B for 5 min was used. Samples were dissolved in ethanol at a concentration of 100 µg ml^{-1} for the extract and 25 - 125 µg ml^{-1} in acetonitrile for coumaryl cinnamate. The autosampler temperature was set at 4°C. The injection volume was 10 µl. Detection was at 272 nm. A calibration curve was used to determine the concentration of coumaryl cinnamate in the extract: 182702x - 57264 (r^2 = 0.9999).

Formulation

For field trials, Siam benzoin petroleum ether extract or Siam benzoin were used in formulations to improve handling, application and properties in the field (e.g. rain fastness). For field trials 2014, the extract was dissolved in a solvent, stabilized and water-diluted before use. For field trials 2015, Siam benzoin was tested as a wettable powder (SB WP; 20% (w/w) Siam benzoin) and an emulsifiable concentrate (SB EC, 15% Siam benzoin resin (w/w)) formulation. A plant oil sticker was added to the spray broth at a final concentration of 0.3%. Blank formulations were tested in semi-controlled bioassays with no or minor effects on the studied plant diseases (data not shown).

Bioassays

1. Pathogens: Phytophthora infestans (Mont.) de Bary was cultivated on V8 agar (200 ml l^{-1} Campbell's V8 or "Biotta® Gemüsecocktail" (vegetable juice) (Biotta AG, Tägerwilen, Switzerland), 3 g l^{-1} $CaCO_3$, 1.5% Agar, pH 6.3) at 20°C in the dark. *Venturia inaequalis* Cooke (Wint.) and *Marssonina coronaria* (Ell. et J.J. Davis) were maintained on apple (*Malus domestica* Borkh.) seedlings cv. 'Jonagold' as described below. Leaves with sporulating lesions were dried at room temperature before storing them in glass vessels at 4°C in the dark. *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni was maintained on grapevine (*Vitis vinifera* L.) seedlings cv. 'Chasselas' by weekly re-inoculation (described below).

2. In Vitro Bioassays:

2.1. General Procedures: All *in vitro* experiments were performed in 96-well plates. Media appropriate for each pathogen were used, namely mineral water ('Evian') for *P. viticola,* demineralised water for *V. inaequalis*, and demineralized water containing 1 ml l^{-1} V8-medium (200 ml l^{-1} Campbell's V8, 3 g l^{-1} $CaCO_3$, pH 6.3) for *P. infestans*. Each test plate contained at least 16 non-treated control wells. The effect of the solvent (DMSO) alone was tested in at least eight replicates in three concentrations per experimental set.

Sporangia suspensions of *P. viticola* (1.8-2.5 x 10^5 sporangia ml^{-1}) and conidia suspensions of *V. inaequalis (*1.5-2.0 x 10^5 conidia ml^{-1}) were prepared by washing fresh (*P. viticola*) or dry (*V. inaequalis*) sporulating leaves with demineralized water. Sporangia suspensions of *P. infestans* (1.2-1.5 x 10^5 sporangia ml^{-1}) were prepared by placing mycelium dispatched from 10-14 d old cultures into demineralized water and shaking vigorously. Suspensions were filtered over a cheese cloth, the concentration was assessed using a Thoma cell counting chamber, and adjusted to desired concentrations.

2.2. Profiling of Microfractions: To determine activity of microfractions against *P. viticola, V. inaequalis, P. infestans,* 6 ul of the test product were added to 96-well plates containing 94 ul of the medium appropriate for each pathogen. Extracts were then serially diluted in the test plate 1:10 and 1:100 by adding 10 ul of the next higher concentration to 90 ul of the appropriate test medium, the 10 ul of the lowest concentration being discarded. Then, 20 ul of a continuously stirred pathogen suspension were added to each well, resulting in extract concentrations of 490, 49 and 4.9 ug ml^{-1}.

2.3. Determination of minimal inhibitory concentrations (MIC_{100}): To determine the concentrations needed to completely inhibit germination of spores or activity of zoospores (MIC_{100}), test products (Siam benzoin petroleum ether extract, Siam benzoin, Sumatra benzoin, coniferyl benzoate or p-coumaryl cinnamate) were dissolved either in DMSO or EtOH (98.9%) at concentrations of 10 mg ml^{-1}. Then, they were serially diluted 1:1 in water down to 0.02 mg ml^{-1} (10 concentrations). 6 ul of each test product and dilution were added to a well containing 94 ul of the appropriate medium before adding 20 ul of pathogen suspension.

2.4 Assessment of inhibitory activity: Effects of extracts were assessed 2-3 h (*P. viticola*), one day (*P. infestans,* bacteria), or two days (*V. inaequalis*) after set-up of the experiment. All assessments were made using a binocular at magnifications X 50 to 100. Inhibition levels were scored according to Table 1.

Table 1. Assessment of inhibition levels caused by plant extracts against *Phytophthora infestans, Venturia inaequalis* and *Plasmopara viticola* in *in vitro* experiments.

Inhibition level	*P. infestans, V. inaequalis*	*P. viticola*
0	Similar to water control	Similar to water control
1	distinct reduction in germination rate and/or length of germ tubes	distinct reduction in number and/or activity of zoospores
2	no germination, or germ tubes ≤ 0.5* length of the sporangium/conidium	no zoospores germinated, or all zoospores inactive

3. Plant-pathogen bioassays under semi-controlled conditions: Plant-pathogen bioassays were carried out under semi-controlled conditions in experimental facilities (greenhouse and growth chambers). Small grapevine (*cv.* 'Chasselas'), apple (*cv.* 'Jonagold') or tomato (*cv.* 'Marmande') seedlings were transplanted to individual pots (0.275 l) containing a standard substrate ('Einheitserde Typ 0', Gebr. Patzer GmbH & Co. KG, Sinntal-Jossa, Germany) previously amended with 3 g l^{-1} of a mineral fertilizer (Tardit 3M, Hauert Günther Düngerwerke GmbH, Erlangen, Germany). Plants were grown in the greenhouse at a temperature of 18 to 28°C under natural light. In wintertime, the photoperiod was extended with mercury lamps to 16 hours. Plants were used for bioassays when they had 2-3 (*P. infestans*), 3-4 (*P. viticola, V. inaeaualis*) or 4-5 fully developed leaves (*M. coronaria*).

Each experimental set included a non-treated non-inoculated control, a water-treated inoculated control, a standard treatment (copper hydroxide, Kocide Opti, DuPont de Nemours, Wilmington, DE, USA) at two concentrations (0.3 g l^{-1} and 0.03 g l^{-1} of Cu^{2+}) (*P. viticola, V. inaequalis, P. infestans*) or two standard treatments (Bordeaux mixture, Bouille bordelaise RSR, Cerexagri S.A., Plaisir, France; 0.6 mg ml-1 Cu^{2+}); Limesulphur, Curatio, Biofa AG, Münsingen, Germany; 6 mg ml^{-1}) *(M. coronaria),* and at least 12 test treatments. All experiments included six replicate plants per treatment. Test products were typically dissolved in DMSO, isopropylidenglycerol or EtOH at concentrations of 50 or 100 mg ml^{-1} and then diluted into water to concentrations between 2 and 0.1 mg ml^{-1}.

Plants were sprayed with the test products using an air-assisted hand sprayer (DeVilbiss® Compact MINI HVLP Touch-Up Spray Gun) or an automatic spray cabinet until leaves (adaxial and abaxial side) were completely covered with a dense layer of small

droplets. Plants were subsequently left to dry at room temperature before inoculation.

P. viticola, *V. inaequalis* and *M. coronaria* inocula were prepared from previously infected plants by washing freshly sporulating grapevine leaves, dried, infected apple leaves (*V. inaequalis*) or dry apple leaves with acervuli (*M. coronaria*) with water and filtering through cheese cloth. *P. infestans* inoculum was prepared from 10-12 d old cultures grown on V8-agar as described above. Concentration of the sporangia/conidia suspensions were adjusted to 5×10^5 sporangia ml^{-1} (*P. viticola*), 7 x 10^5 conidia ml^{-1} (*V. inaequalis*), 1.5-2 x 10^5 sporangia ml^{-1} (*M. coronaria*) or 5 x 10^4 sporangia ml^{-1} (*P. infestans*), respectively. Plants were spray-incoculated using an air-assisted hand sprayer on the the abaxial (*P. viticola*) or the adaxial (*V. inaequalis, M. coronaria, P. infestans*) leaf side. Inoculated plants were subsequently incubated at 20–21°C and 80–99% of relative humidity (RH) in the light for 24 h (*P. viticola, V. inaequalis, P. infestans*) or for a minimum of 72 h with a 16/8-h day/night light regime (*M. coronaria*). Then, plants were maintained at 20°C, 60–80% RH, and a 16/8-h day/night light regime. For grapevine bioassays, 5 to 6 d after inoculation, plants were incubated over night in the dark at 20°C and 80–99% to promote sporulation. Disease incidence (percentage of leaves with disease symptoms) and disease severity (percentage of leaf area covered by lesions) were assessed 5 d (*P. infestans*) 6 to 7 d (*P. viticola*), 10 to 12 d (*V. inaequalis*) or 14 d (*M. coronaria*) after inoculation. Disease assessments for all pathogens except *M. coronaria* were made using continuous values of percentage based on the EPPO standard scale. For *M. coronaria*, disease severity of each individual leaf was categorized into one of five classes (0: no disease symptoms, 1: 1-5 spots per leaf; 2: 6-20 spots per leaf; 3: 21-50 spots per leaf; 4: >50 spots per leaf) 14 d after inoculation. The relative frequency of each disease class was calculated per plant.

Field Trials

1. General procedures: Efficacy of Siam benzoin against downy mildew (*Plasmopara viticola*) and powdery mildew (*Oidium tuckeri*) was tested under field conditions (natural infections, no artificial inoculation). The experiments were conducted following EPPO guidelines (PP1/031(1) *Plasmopara viticola*; PP1/152(4) Design and Analyses of Efficacy Trials; PP1/181(4) Conduct and Reporting of Efficacy Trials-GEP; PP1/135(3) Phytotoxicity assessment) (pp1.eppo.int/list.php). In 2015, experiments were performed under GEP.

2. Experimental vineyard: The experiment was carried out in the screening-vineyard of the Research Institute of Organic Agriculture in Frick, Switzerland, at 385 meters a.s.l, on a

clayey loam. The coordinates of the experimental plot are: 47°31’4’’ N 08°01’33’’ E. Average annual rainfall is 1138 mm (mean 2005-2014, www.agrometeo.ch). The experimental vineyard was established in 1997 and consists of 576 plants of the susceptible grapevine varieties ‘Müller-Thurgau’ (‘Riesling x Sylvaner’) and ‘Chasselas’ (‘Gutedel’) (288 plants per variety). Plant distance between rows is 2 m, within rows 1.1 m (4545 plants/ha). The experiment was set up in a complete randomized block design, with twelve treatments arranged in 4 replicates of 6 plants for both grapevine varieties. Due to the age of the vineyard and its previous use in trials there are some plants of low quality. This resulted in some replicates having less than 12 plants. The vineyard was maintained according to guidelines for organic agriculture. Maintenance work included fertilization with an organic fertilizer and budding treatment with sulphur against mites in April, thinning of shoots, shortening of main and secondary shoots and grape zone defoliation.

Weather data were recorded throughout the season with a Campbell weather station close to the vineyard (www.agrometeo.ch).

3. Test products: As a copper control, Kocide Opti (copper hydroxide, DuPont de Nemours, Wilmington, DE, USA) was used at a final concentration of 0.1% of the product (containing 0.03% Cu^{2+}).‘Strategy Praxis’ is the plant protection strategy recommended by the FiBL-advisory service to Swiss grapevine producers. The spray schedule starts with the use of ‘Mycosin’ (Andermatt Biocontrol, Grossdietwil, Switzerland; containing 65% acidified clay minerals, 0.2% horsetail extract, concentration of formulation 0.8%) plus ‘Stulln Sulphur’ (Andermatt Biocontrol, 80% sulphur, concentration of formulation 0.5%) in tank mixture. Around bloom, depending on infection pressure and rainfall, there is a change to copper (Kocide opti, 0.1%), which is sprayed until the end of the season. In 2014 and 2015, the change to copper was on 5 July 2014 or 22 June 2015 respectively, after 8 (2014) or 6 (2015) copper-free treatments. An untreated control serves as a reference for natural development of disease epidemic.

In 2014, Siam benzoin petroleum ether extract (“SB-PE”) was tested at a concentration of 1 g l^{-1}. The extract was dissolved in a solvent before adding a stabilizer and dilution into water. In 2015, Siam benzoin was tested in two preliminary formulations, a wettable powder (SB WP) and an emulsifiable concentrate (SB EC). Both formulations were based on Siam benzoin and applied at a final concentration of 1 g $l^{-1}$of the resin.

4. Applications: Products were applied by hand using two pressure based and pressure tank supported spray systems (spray gun: GTi Pro light pressure, DeVillbiss, USA; pressure

tank: pressure feed cup KB-522-SS, DeVillbiss, USA; 4 bar spray pressure). The two spray systems were calibrated to dispense similar amounts of product per unit of time.

Plants were treated by spraying the product from above and from below, which resulted in a homogeneous coating of the abaxial and adaxial leaf surface. Spray distribution was verified using water-sensitive paper (Novartis, Basel, Switzerland).

Plants were treated weekly or according to weather conditions and risk for infection, calculated by the forecast model 'vitimeteo' (www.agrometeo.ch). Treatments started 6 May (2014) or 13 May (2015) and ended on 20 (2014) or 21 (2015) August. In both years, a total of 16 treatments were performed in intervals of 3 to 10 days.

5. Disease assessments

5.1 Plasmopara viticola (downy mildew): Three (5, 19 and 27 August 2014) or four (26 June, 3 July, 23 July, 18 August 2015) disease assessments were carried out by scoring disease incidence (proportion of leaves with symptoms) and disease severity (proportion of diseased leaf area) of *Plasmopara viticola* (downy mildew) on leaves (assessment of 100 or all leaves per plant, all plants per variety and replication). In 2014, overall damage on grapes caused by downy and powdery mildew was assessed 9 September 2014 for each treatment replicate. In 2015, percentage grape area infected by *P. viticola* was assessed 24 July 2015 (assessment of all grapes per plant, all plants per variety and replication).

5.2 Oidium tuckeri (powdery mildew): Oidium tuckeri on leaves was assessed once per season. In 2014, the percentage leaves with infections and the infected leaf area was assessed on 50 leaves per plant 11 August 2014. In 2015, powdery mildew disease severity on leaves was categorized into four classes (0-3) (assessment of all leaves per plant, all plants per variety and replication) 21 August 2015. In 2014, percentage grapes infected by powdery mildew was assessed 24 July 2014 (assessment of all grapes per plant, all plants per variety and replication). In 2015, presence/absence of *O. tuckeri* on grapes was evaluated for each plant (24 July 2015).

Calculations and statistical analyses

To calculate means and confidence intervals of MIC_{100} values, data were log_2-transformed. 95% confidence intervals were calculated from transformed data as $A \pm 1.96*B*n^{-0.5}$, with A = mean MIC_{100}, B = standard deviation MIC_{100} and n = number of experiments. Data were transformed back to the linear scale for presentation in tables.

To check for a treatment effect in field trials, a one-way analysis of variance and a comparison between the replicate means of all treatments was done by using Tukey's HSD (Honestly Significant Difference) test. Prior to all analysis, all data was arcsin-transformed (not shown).

Efficacies were calculated according to Abbott as (1- ($A*B^{-1}$)) * 100. In semi-controlled bioassays, A is disease severity/incidence on an individual plant and B mean disease severity/incidence of control plants. In field experiments, A is the mean disease severity/incidence of a treatment and B is the mean disease severity/incidence of the non-treated control.

EXAMPLE 1

***In vitro* activity of Siam and Sumatra benzoin and identification of the active ingredients**

The petroleum ether extract of Siam benzoin ('SB-PE') showed strong activity against *Plasmopara viticola*, *Phytophthora infestans*, and *Venturia inaequalis* in *in vitro* bioassays, with minimal inhibitory concentrations (MIC_{100}) of 26 µg ml^{-1} (*P. viticola*), 45 µg ml^{-1} (*V. inaequalis*) and 32 µg ml^{-1} (*P. infestans*) (Table 2). Similar results were found when Siam benzoin (SB) was directly dissolved in EtOH ("SB-EtOH") (Table 2). MIC_{100} of Sumatra benzoin dissolved in EtOH ("SumB-EtOH") were between 48 µg ml^{-1} (*P. viticola*) and 99 µg ml^{-1} (*V. inaequalis*).

Table 2. Minimal inhibitory concentrations (MIC_{100}) of Siam benzoin petroleum ether extract (SB-PE), Siam benzoin (SB-EtOH), Sumatra benzoin (SumB-EtOH) dissolved in EtOH, coniferyl benzoate (CB) and p-coumaryl cinnamate (CC) against *Plasmopara viticola, Venturia inaequalis* and *Phytophthora infestans*. The table shows means (bold), lower and upper limits of the 95% confidence interval (in brackets) and number of independent experiments (N).

	P. viticola		***V. inaequalis***		***P. infestans***	
	Mean[a]	N	Mean[a]	N	Mean[a]	N
SB-PE	**26** (13; 49)[b]	8	**45** (24;78)[b]	8	**32** (8; 121)[b]	3
SB-EtOH	**14** (12; 18)	2	**63** (17;240)	2	**32** (32; 32)	2

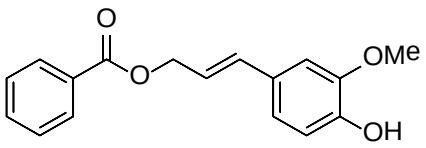
SumB-EtOH	**48** (32;73)	3	**99** (46;156)	3	**79** (32;195)	3
CB	**12** (6;22)	7	**35** (23;54)	7	**21** (15;29)	5
CC	**38** (27;54**)**	3	**32** (15;70)	3	**25** (16;40)	3

[a] μg ml^{-1}
[b] upper and lower limit of the 95% confidence interval in μg ml^{-1}

The active ingredient of Siam benzoin was identified by a process referred to as HPLC-based activity profiling. An aliquot of the petroleum ether extract was separated by semi-preparative HPLC and each fraction was tested *in vitro* against three plant pathogens. When the bioactivity data and the chromatographic trace were compared the activity could be mainly assigned to Fraction 14, eluting between 20.5 and 22.0 min (data not shown). This fraction contained a major peak which was isolated by preparative HPLC. It was identified by comprehensive NMR analysis as coniferyl benzoate (Compound **1**). Coniferyl benzoate (CB) was subsequently quantified by HPLC-UV analysis and found to account for 43-53% of the petroleum ether extract, and 33-59% in the EtOH soluble part of the SB resin.

The HPLC chromatogram of Sumatra benzoin showed a major UV peak with a similar elution time as CB. After isolation, it was identified as p-coumaryl cinnamate (Compound **2**), a chemically related ester. Quantitative analysis by HPLC-UV revealed that this compound made up 27-29% of SumB resin.

Minimal inhibitory concentrations MIC_{100} of coniferyl benzoate against *P. viticola, V. inaequalis* and *P. infestans* ranged between 8 and 32 μg ml^{-1} (Table 2). MIC_{100} of p-coumaryl cinnamate (CC) were slightly higher, ranging between 16 and 64 μg ml^{-1}.



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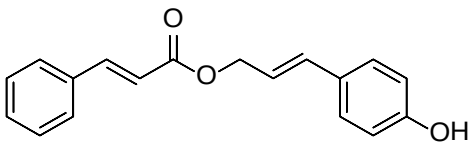

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EXAMPLE 2

Fungicidal activity of Siam and Sumatra benzoin and their active ingredients on grapevine, apple and tomato seedlings under semi-controlled conditions

2.1. Grapevine – *P. viticola*: Efficacy of Siam benzoin petroleum ether extract on grapevine seedlings against *P. viticola* under semi-controlled conditions was very high. At an application rate of 1 mg ml^{-1}, the diseased leaf area was reduced by 99% compared to the non-

treated control (i.e. 99% efficacy) in three out of four independent experiments and by 81% in a fourth experiment (Table 3). At 0.25 mg ml^{-1}, mean efficacy was still 75%. Efficacies of Siam benzoin and of Sumatra benzoin dissolved in EtOH (SB-EtOH, SumB-EtOH) were comparable (Table 4). Efficacies of purified coniferyl benzoate (CB) and p-coumaryl cinnamate (CC) were ≥98% at 1 mg ml^{-1} and ≥80% at 0.25 mg ml^{-1} (Table 5).

Table 3. Efficacy of Siam benzoin petroleum ether extract (SB-PE) (1 and 0.25 mg ml^{-1}) on grapevine seedlings against *P. viticola* under semi-controlled conditions, compared to efficacies of two concentrations of a copper reference treatment (Cu^{2+} in the form of copper hydroxide, Kocide Opti®). The table shows results (means ±SD) of four (SB-PE 1 mg ml^{-1}) or two (0.25 mg ml^{-1}) independent experiments, each experiment with 6 replicate plants per treatment and concentration.

	Efficacy (%) [1]				Disease severity non-treated control (%) [2]
Product	SB-PE		Cu^{2+}		
Concentration (mg ml^{-1})	1	0.25	0.3	0.03	
Exp_1	81 ± 15		100 ± 0	76 ± 17	65 ± 16
Exp_2	99 ± 1	60 ± 9	99.6 ± 0.7	99.4 ± 0.5	77 ± 22
Exp_3	99 ± 1	90 ± 9	100 ± 0	95 ± 5	36 ± 18
Exp_4	99 ± 1		98 ± 2	89 ± 10	78 ± 5
Mean [3]	**95 ± 9**	**75 ± 21**	**99 ± 1**	**90 ± 10**	**64 ± 20**

[1] Percentage reduction in the diseased leaf area in treated plants compared to the non-treated control
[2] Percentage leaf area with disease symptoms; [3] Mean and SD of all independent experiments

Table 4. Efficacy of Siam benzoin (SB-EtOH) and Sumatra benzoin (SumB-EtOH) dissolved in EtOH and a copper reference (Cu^{2+} in the form of copper hydroxide, Kocide Opti®) against *Plasmopara viticola* on grapevine cv. 'Chasselas' seedlings under semi-controlled conditions. The table shows means ± SD (n = 6). Disease severity (percentage leaf area with disease symptoms) of the non-treated control was 82% ± 22%.

Treatment	Concentration (mg ml^{-1})	Efficacy Mean± SD
Cu^{2+}	0.3	97±2
	0.03	90±7

SB-EtOH	2	98±5
	1	100±0
	0.5	92±12
	0.25	87±9
	0.125	41±28
SumB-EtOH	2	99±1
	1	100±0
	0.5	96±2
	0.25	92±13
	0.125	81±9

Table 5. Efficacy of coniferyl benzoate (CB) and p-coumaryl cinnamate (CC) against *P. viticola* on grapevine cv. 'Chasselas' seedlings under semi-controlled conditions. Each experimental set included a copper reference (Cu^{2+} in the form of copper hydroxide, Kocide Opti®). The table show means ± SD (n = 6).

Concentration	CB[1]	CC[2]
1 mg ml^{-1}	99±1	98±1
0.5 mg ml^{-1}	77±50	95±6
0.25 mg ml^{-1}	85±14	80±16

[1] Disease severity control 49% ±18 %, efficacy Cu^{2+} 100%±0% (0.3 gm ml^{-1}) and 99%±2% (0.03 mg mL^{-1}); [2] Disease severity control 82% ± 22%, efficacy Cu^{2+} 97%±2 % (0.3 mg ml^{-1}) and 90±7 (0.03 mg ml^{-1})

2.2. Apple – *V. inaequalis*: Efficacy of Siam benzoin petroleum ether extract on apple seedlings against *V. inaequalis* under semi-controlled conditions was between 95% and 99% at 2 mg ml^{-1} and 83% and 95% at 1 mg ml^{-1} (Table 6). Efficacy of coniferyl benzoate at 1 mg ml^{-1} was 70% (Table 7).

Table 6. Efficacy of Siam benzoin petroleum ether extract (SB-PE) (1 and 2 mg ml^{-1}) on apple seedlings *cv.* 'Jonagold' against *V. inaequalis* under semi-controlled conditions, compared to efficacies of two concentrations of a copper reference treatment (Cu^{2+} in the form of copper hydroxide, Kocide Opti®). The table shows results (means ±SD) of three independent experiments for each concentration of SB-PE, each experiment with 6 replicate plants per treatment and concentration.

Efficacy (%) [1]	Disease severity non-treated control

Product	SB-PE		Cu^{2+}		(%) [2]
Concentration (mg ml^{-1})	2	1	0.3	0.03	
Exp_1		83 ± 8	86 ± 12	62 ± 18	23 ± 14
Exp_2 [4]	99 ± 2	95 ± 3	87 ± 9	84 ± 15	28 ± 14
Exp_3 [4]	99 ± 2		97 ± 2	87 ± 7	13 ± 4
Exp_4 [4]	95 ± 4	88 ± 8	92 ± 4	77 ± 18	33 ± 8
Mean [3]	**98 ± 2**	**89 ± 6**	**91 ± 5**	**78 ± 11**	**24 ± 9**

[1] Percentage reduction in the diseased leaf area in treated plants compared to the non-treated control; [2] Percentage leaf area with disease symptoms; [3] Mean and SD of all independent experiments, [4] Siam benzoin petroleum ether extract in a formulation

Table 7. Efficacy of coniferyl benzoate (CB) and a copper reference (Cu^{2+} in the form of copper hydroxide, Kocide Opti®) against *Venturia inaequalis* on apple cv. 'Jonagold' seedlings under semi-controlled conditions. The table shows means ± SD (n = 6). Disease severity (percentage leaf area with disease symptoms) of the non-treated control was 23 % ± 14 %.

	Concentration	Efficacy Mean±SD
CB	1 mg ml^{-1}	69±12
	0.1 mg ml^{-1}	4±53
Cu^{2+}	0.3 mg ml^{-1}	85±12
	0.03 mg ml^{-1}	62±18

Apple – *D. mali*

Siam benzoin significantly reduced Marssonina leaf drop caused by *M. coronaria* on apple seedlings compared to the non-treated control (Tab. 8). The percentage of leaves without any disease symptoms (disease category 0) or with few symptoms (category 1) was 83-98% in SB-treated plants compared to 11% in control plants ($p<0.05$, Tukey-B). As a consequence, percentage of leaves with more severe symptoms (categories 2-4) was significantly reduced in SB-treated plants compared to the control. One formulation (SB EC-2) at a concentration of 7.5 mg ml^{-1} was even comparable to Limesulphur, the best organic reference treatment.

Table 8. Efficacy of two formulations of Siam benzoin (SB EC-1 and SB EC-2) at two concentrations (2.5 and 7.5 mg ml^{-1}) on apple seedlings *cv.* 'Jonagold' against *M. coronaria* under semi-controlled conditions, compared to efficacies of a copper (Bordeaux mixture, 0.6

mg ml^{-1} Cu^{2+}) and a Limesulphur reference (6 mg ml^{-1}). Disease levels were categorized into 5 classes (Cat. 0: no disease; Cat. 1: 1-5 spots per leaf; Cat. 2: 6-20 spots per leaf, Cat. 3: 21-50 spots per leaf; Cat. 4: >50 spots per leaf) and the mean relative frequency of each disease class was calculated per plant. The table shows means of the six replicate plants per treatment. Different letters indicate significant differences between treatments of a category (Tukey-B, $p<0.05$).

Treatment	Mean					SD				
	Cat 0	Cat 1	Cat 2	Cat 3	Cat 4	Cat 0	Cat 1	Cat 2	Cat 3	Cat 4
Control	1 a	10 a	50 a	32 a	7 a	4	13	13	16	9
Bordeaux mixture	11 ab	47 bc	38 ac	4 b	0 a	12	23	26	5	0
Limesulphur	75 d	25 ab	0 b	0 b	0 a	7	7	0	0	0
SB EC-1 7.5 mg ml^{-1}	35 bc	63 c	2 b	0 b	0 a	16	14	4	0	0
SB EC-1 2.5 mg ml^{-1}	23 bc	60 c	17 bc	0 b	0 a	20	20	15	0	0
SB EC-2 7.5 mg ml^{-1}	54 cd	44 abc	2 b	0 b	0 a	33	29	5	0	0
SB EC-2 2.5 mg ml^{-1}	22 bc	68 c	11 bc	0 b	0 a	21	16	11	0	0

2.3. Tomato – *P. infestans*. Efficacy of Siam benzoin petroleum ether extract on tomato seedlings against *P. infestans* under semi-controlled conditions ranged between 89% and 100% at 2 mg ml^{-1} and 63% and 100% at 1 mg ml^{-1} (Table 9), and was even superior to the copper reference.

Table 9. Efficacy of Siam benzoin petroleum ether extract (SB-PE) (1 and 2 mg/ml) on tomato seedlings (*cv.* 'Marmande') against *P. infestans* under semi-controlled conditions, compared to efficacies of two concentrations of a copper reference treatment (Cu^{2+} in the form of copper hydroxide, Kocide Opti®). The table shows results (means ± SD) of four independent experiments, each experiment with 6 replicate plants per treatment.

	Efficacy (%) [1]				Disease severity non-treated control (%) [2]
Product	SB-PE		Cu^{2+}		
Concentration (mg ml^{-1})	2	1	0.3	0.03	
Exp_1	89 ± 20		87 ±12	57 ± 25	67 ± 10

Exp_2	92 ± 4	63 ± 21	75 ± 23	59 ± 28	85 ± 9
Exp_3	100 ± 0	100 ± 0	72 ± 23	75 ± 17	93 ± 13
Exp_4	93 ± 10	87 ± 10	95 ± 6	63 ± 16	68 ± 5
Mean [3]	**94 ± 5**	**83 ± 19**	**82 ± 11**	**64 ± 8**	**78 ± 13**

[1] Percentage reduction in the diseased leaf area in treated plants compared to the non-treated control; [2] Percentage leaf area with disease symptoms; [3] Mean and SD of all independent experiments

EXAMPLE 3

Fungicidal activity of Siam benzoin on grapevine against downy mildew caused by *P. viticola* and powdery mildew caused by *Oidium tuckeri* under field conditions

3.1 Downy mildew (P. viticola)

3.1.1. Disease development: In 2014, disease pressure of downy mildew caused by *P. viticola* was relatively low in the primary season due to the warm and dry wheather conditions from Mid-May until end of June. The first major infection period for downy mildew occurred at the beginning of June 2014 and resulted in few first lesions in Mid-June. During August 2014, downy mildew developed rapidly such that by the end of August, disease incidence was up to 100% and severity about 50% (Table 10). Treatments with a high incidence of powdery mildew showed a low rate of downy mildew sporulation on leaves. In these cases the symptoms directly developed into mosaic- and later necrotic spots.

In 2015, the first visible downy mildew symptoms appeared in the beginning of June. Until the end of June 2015, the degree of infection progressed quite rapidly. The warm and dry weather conditions during July and August 2015 slowed down the infection progress. By the end of August 2015, the disease incidence reached approx. 50% and disease severity approx. 9% in the untreated control (Table 11).

3.1.2 Efficacy of test products

In both seasons, the standard fungicide program as recommended for Swiss organic grapevine production as well as the copper control protected leaves and fruit very efficiently from downy and powdery mildew (>92% efficacy with 16 treatments).

In both seasons, Siam benzoin showed a significant effect against downy mildew at the end of the season. In 2014, Siam benzoin petroleum ether extract (SB-PE) reduced disease

severity on leaves by 31% at the end of the season (Table 10). In 2015, Siam benzoin (SB) reduced downy mildew disease incidence and severity compared to the non-treated control throughout the whole season, and differences became significant on the last disease assessment mid of August, with efficacies between 64% and 68% (Table 11). On grapes, downy mildew disease severity was reduced by Siam benzoin up to 87% in 2015 (Table 12). In 2014, late occurrence of downy mildew (after powdery mildew infections) did not allow for a downy mildew disease assessment on grapes. Yet, overall damage on grapes caused by both diseases was reduced by 43% on plants treated with Siam benzoin petroleum ether extract as compared to non-treated control plants (Table 13).

Table 10: Disease development (severity) of downy mildew caused by *P. viticola* under field conditions in 2014 on leaves of untreated grapevine plants (Control) and plants treated with Siam benzoin petroleum ether extract (SB-PE) (1 g l^{-1}), copper (0.3 g l^{-1} Cu^{2+} in the form of copper hydroxide, Kocide Opti®) or a plant protection strategy recommended by the FiBL-advisory service to Swiss grapevine producers ('Strategy'). Disease severity was assessed on three dates: 5 August 2014 (Table 10A), 19 August (Table 10B), 27 August (Table 10C).

10A

	05.08.2014			
	Severity (%)		Efficacy (%) [c]	Tukey-B [b]
Treatment	Mean [a]	SD [a]		
untreated	4.1	2.0	-	A
copper	0.1	0.0	97.7	B
ref. strategy	0.1	0.1	97.6	B
SB-PE	2.7	0.9	32.2	A

10B

	19.08.2014			
	Severity (%)		Efficacy (%) [c]	Tukey-B [b]
Treatment	Mean [a]	SD [a]		
untreated	22.3	5.7	-	A
copper	1.3	0.4	94.0	B
ref. strategy	1.9	1.0	91.6	B
SB-PE	12.6	1.3	43.7	C

10C

	27.08.2014			
	Severity (%)		Efficacy (%) [c]	Tukey-B [b]
Treatment	Mean [a]	SD [a]		
untreated	50.7	10.8	-	A
copper	3.1	0.7	93.9	B
ref. strategy	3.4	1.4	93.4	B
SB-PE	35.2	6.3	30.5	C

[a] means and standard deviations (SD) of four treatment replicates.

[b] Different letters indicate significant differences between treatments

[c] Efficacy calculated according to Abbott in %

Table 11: Disease development (severity) of downy mildew caused by *P. viticola* under field conditions in 2014 on leaves of untreated grapevine plants (Control) and plants treated with copper (0.3 g l^{-1} Cu^{2+} in the form of copper hydroxide, Kocide Opti®), a plant protection strategy recommended by the FiBL-advisory service to Swiss grapevine producers ('Strategy') or two formulations based on Siam benzoin resin (SB WP and SB EC) (2 g l^{-1} extract). Disease severity was assessed on four dates: 26 June 2015 (Table 11A), 3 July 2015 (Table 11B), 24 July 2015 (Table 11C), and 18 August 2015 (Table 11D).

11A

	26.06.2015			
Treatment	Severity (%)		Efficacy (%) [c]	Tukey-B [b]
	Mean [a]	SD [a]		
Control	2.8	4.7		A
Copper	0.3	0.3	90.0	A
Strategy	0.2	0.2	93.9	A
SB WP	1.4	1.7	51.1	A
SB EC	1.0	0.9	65.7	A

11B

	03.07.2015			
Treatment	Severity (%)		Efficacy (%) [c]	Tukey-B [b]
	Mean [a]	SD [a]		
Control	3.0	3.1	0.0	A
Copper	0.6	0.3	79.9	B
Strategy	0.6	0.5	81.0	B
SB WP	1.5	0.9	50.3	AB
SB EC	1.1	0.6	61.8	AB

11C

Treatment	24.07.2015			
	Severity (%)		Efficacy (%) [c]	Tukey-B [b]
	Mean [a]	SD [a]		
Control	5.0	4.0		A
Copper	1.2	0.6	75.8	BC
Strategy	0.9	0.6	82.6	B
SB WP	3.5	1.7	30.1	AC
SB EC	2.5	1.3	49.2	ABC

11D

Treatment	18.08.2015			
	Severity (%)		Efficacy (%) [c]	Tukey-B [b]
	Mean [a]	SD [a]		
Control	8.7	5.9		A
Copper	1.0	0.4	88.3	B
Strategy	0.9	0.6	89.9	B
SB WP	3.1	2.1	64.2	B
SB EC	2.8	1.8	68.1	B

[a] means and standard deviations (SD) of four treatment replicates.

[b] Different letters indicate significant differences between treatments

[c] Efficacy calculated according to Abbott in %

Table 12. Downy mildew disease severity under field conditions in 2015 on grapes of non-treated plants (Control) and on grapes of plants treated with copper (0.3 g l^{-1} Cu^{2+} in the form of copper hydroxide, Kocide Opti®), a plant protection strategy recommended by the FiBL-advisory service to Swiss grapevine producers ('Strategy') or two formulations based on Siam benzoin (SB WP and SB EC) (2 g l^{-1} Siam benzoin).

	Incidence (%)				Severity (%)			
	Mean[a]	SD[a]	Tukey[b]	Efficacy (%) [c]	Mean[a]	SD[a]	Tukey[b]	Efficacy (%) [c]
Control	51.4	25	B		19.9	22.7	B	
Copper	20.6	11.3	AB	59.9	1.2	1.3	A	93.9
Strategy	9.3	6.8	A	81.9	1.8	3.4	A	90.7
SB WP	8.4	5.7	A	83.6	0.8	1.3	A	96.2
SB EC	18.3	14.9	AB	64.3	2.5	3.4	AB	87.2

[a] means and standard deviations (SD) of four treatment replicates.

[b] Different letters indicate significant differences between treatments

[c] Efficacy calculated according to Abbott in %

Table 13. Overall damage caused by downy and powdery mildew under field conditions in 2014 on grapes of non-treated grapevine plants (Control) and on plants treated with copper (0.3 g l^{-1} Cu^{2+} in the form of copper hydroxide, Kocide Opti®), a plant protection strategy recommended by the FiBL-advisory service to Swiss grapevine producers ('Strategy') or a Siam benzoin petroleum ether extract (SB-PE) (1 g l^{-1}).

	Severity (%)			
	Mean[a]	SD[a]	Tukey[b]	Efficacy
Control	76.3	21.6	A	
Copper	3.3	1.1	B	95.7
Strategy	3.8	1.3	B	95.1
SB-PE	43.8	19.8	C	42.6

[a] means and standard deviations (SD) of four treatment replicates.

[b] Different letters indicate significant differences between treatments

[c] Efficacy calculated according to Abbott in %

*3.2 Powdery mildew (*Oidium tuckeri*)*

3.2.1 Disease development: In both seasons (2014 and 2015), there was unusually high disease pressure by powdery mildew caused by *Oidium tuckeri*, resulting in 67% (2014) or approx. 30% (2015) powdery mildew disease severity on leaves and 56% (2014) or 60% (2015) disease incidence on grapes (Tables 14A and 14B).

3.2.2 Efficacy of test products: In both seasons, the standard fungicide program as recommended for Swiss organic grapevine production as well as the copper control protected leaves and fruit very efficiently from powdery mildew (>90% efficacy) (Tables 14A and 14B).

In both seasons, SB-PEshowed good efficacy against powdery mildew. Powdery mildew disease severity on leaves was reduced by 66% in 2014 (Table 14A) and by 75 and 86% in 2015 (Table 14B), while efficacy on grapes was 51% in 2014 and 30% in 2015.

Table 14A. Powdery mildew disease under field conditions in 2014 (on leaves and grapes of non-treated plants (Control) and plants treated with copper (0.3 g l^{-1} Cu^{2+} in the form of copper hydroxide, Kocide Opti®), a plant protection strategy recommended by the FiBL-advisory service to Swiss grapevine producers ('Strategy') or by Siam benzoin (Siam benzoin petroleum ether extract, 1 g l^{-1}).

	2014							
	Leaves Severity (%)				Grapes (Incidence %)			
	Mean[a]	SD[a]	Tukey[b]	Efficacy[c]	Mean[a]	SD[a]	Tukey[b]	Efficacy[c]
Control	67.3	11.7	A		55.7	21.0	B	-
Copper	0.0	0.0	B	100.0	4.6	4.3	A	91.8
Strategy	0.0	0.0	B	100.0	3.5	0.6	A	93.7
SB-PE	23.0	17.5	C	65.8	27.5	24.5	AB	50.7

[a] means and standard deviations (SD) of four treatment replicates.

[b] Different letters indicate significant differences between treatments

[c] Efficacy calculated according to Abbott in %

Table 14B. Powdery mildew disease under field conditions in 2015 (on leaves and grapes of non-treated plants (Control) and plants treated with copper (0.3 g l^{-1} Cu^{2+} in the form of copper hydroxide, Kocide Opti®), a plant protection strategy recommended by the FiBL-advisory service to Swiss grapevine producers ('Strategy') or by Siam benzoin (two formulations of Siam benzoin: SB WP and SB EC, 2 g l^{-1} Siam benzoin).

	2015							
	Severity classes (0-3)[d]				Grapes (Incidence %)			
	Mean[a]	SD[a]	Tukey[b]	Efficacy[c]	Mean[a]	SD[a]	Tukey[b]	Efficacy[c]
Control	2.6	0.3	A		60.0	19.6	B	
Copper	0.0	0.0	B	100.0	15.4	10.8	AB	74.3
Strategy	0.1	0.3	BC	95.1	18.3	21.3	AB	69.5
SB WP	0.4	0.2	BC	86.3	41.7	44.1	AB	30.5
SB EC	0.6	0.3	C	74.8	42.9	24.0	AB	28.5

[a] means and standard deviations (SD) of four treatment replicates.

[b] Different letters indicate significant differences between treatments

[c] Efficacy calculated according to Abbott in %

[d] four disease classes: 0: no symptoms; 1: low disease level (<10% of leaves show 1-2 colonies); 2: intermediate disease level (> 10% of leaves show symptoms/ more than 2 colonies on leaves); 3: high disease level (predominantly old necrotic spots / symptoms through all leaf ages).

EXAMPLE 4

Fungicidal activity of Balsam of Peru *in vitro* and on grapevine and apple seedlings against *P. viticola* and *V. inaequalis* and identification of the active ingredient

MIC_{100} of Balsam of Peru *in vitro* was 16 μg ml^{-1} against *P. viticola* and was thus comparable to Siam benzoin (SB) and Sumatra benzoin (SumB) (Tab. 2). MIC_{100} against *V.*

inaequalis and *P. infestans* were between 250 and 500 μg ml^{-1}. Balsam of Peru efficiently protected grapevine seedlings against *P. viticola* (efficacy of 90% at a concentration of 1 mg ml^{-1}) and apple seedlings against *V. inaequalis* (efficacy of 97% at 2.5 mg ml^{-1}) (Tab. 16). Efficacy was comparable to a copper reference.

HPLC analysis of Balsam of Peru showed the presence of a major peak in the UV chromatogram. The compound was isolated by preparative HPLC and identified as benzyl cinnamate (compound 3) by comprehensive NMR analysis, and ESI mass spectrometry. Benzyl cinnamate (BC) was very active against *P. viticola* in *in vitro* experiments, with MIC_{100} of 8 μg ml^{-1} (Tab. 15).

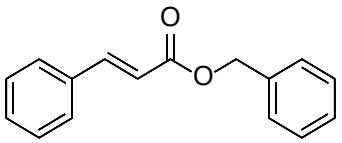

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Table 15. Minimal inhibitory concentrations (MIC_{100}) of Balsam of Peru (BP) and benzyl cinnamate (BC) against *Plasmopara viticola, Venturia inaequalis* and *Phytophthora infestans*.

	MIC_{100}		
	P. viticola	*V. inaequalis*	*P. infestans*
BP	16[a]	500	250
BC	8	>500	500

[a] μg ml^{-1}

Table 16. Efficacy of Balsam of Peru dissolved in DMSO (BP-DMSO) and a copper reference (Cu^{2+} in the form of copper hydroxide, Kocide Opti®) against *Plasmopara viticola* on grapevine cv. 'Chasselas' seedlings and against *Venturia inaequalis* on apple cv. 'Jonagold' seedlings under semi-controlled conditions. The table shows means ± SD (n = 6). Disease severity (percentage leaf area with disease symptoms) of the non-treated control was 84% ± 12% (*P. viticola*) or 20% ± 12% (*V. inaequalis*)

Treatment	Conc (mg ml^{-1})	Efficacy (%) ± SD	
		P. viticola	*V. inaequalis*
Cu^{2+}	0.3	87 ± 9	91 ± 6
	0.03	52 ± 24	50 ± 43
BP-DMSO	2.5	90 ± 12	97 ± 5
	1	90 ± 9	57 ± 61
	0.25	18 ± 19	19 ± 69

REFERENCES

Dagostin S., Formolo T., Giovannini O., Pertot I., Schmitt A. (2010) *Salvia officinalis* extract can protect grapevine against *Plasmopara viticola*. Plant disease 94:575-580.

Hovaneissian M., Archier P., Mathe C., Culioli G., Vieillescazes C. (2008) Analytical investigation of styrax and benzoin balsams by HPLC-PAD-fluorimetry and GC-MS. Phytochemical Analysis 19:301-310.

Isman M.B., Akhtar Y. (2007) Plant natural products as a source for developing environmentally acceptable insecticides, in: I. Ishaaya, et al. (Eds.), Insecticides design using advanced technologies, Springer-Verlag, Berlin Heidelberg. pp. 235-248.

Scherf A., Treutwein J., Kleeberg H., Schmitt A. (2012) Efficacy of leaf extract fractions of *Glycyrrhiza glabra* L. against downy mildew of cucumber (*Pseudoperonospora cubensis*). Eur. J. Plant Pathol. 134:755-762. DOI: 10.1007/s10658-012-0051-0.

Speiser B., Tamm L., Roggli M., Berner A., Bickel R., Maurer V., Schneider C., Chevillat V. (2016) Betriebsmittelliste 2016 Research Institute of Organic Agriculture, Frick.

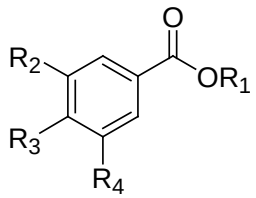
Tamm L., Speiser B. (2015) Direct control of airborne diseases, in: M. R. Finckh, et al. (Eds.), Plant diseases and their management in organic agriculture, APS Press, St. Paul, Minnesota, USA.

Van-Zwieten L., Merrington G., Van-Zwieten M. (2004) Review of impacts on soil biota caused by copper residues from fungicide application. SuperSoil 2004:3rd.

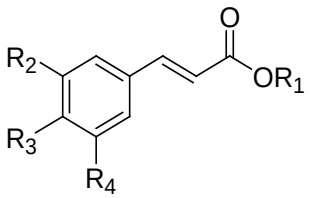
Vogt V., Cifuente D., Tonn C., Sabini L., Rosas S. (2013) Antifungal activity *in vitro* and *in vivo* of extracts and lignans isolated from *Larrea divaricata* Cav. against phytopathogenic fungus. Ind. Crops Prod. 42:583-586. DOI: http://dx.doi.org/10.1016/j.indcrop.2012.06.009.

CLAIMS

1. Use of a composition as a fungicide, wherein said composition comprises at least one compound of formula (I) or formula (II)

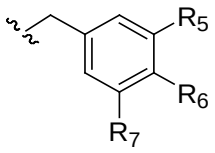
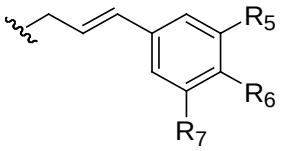


(I)



(II)

wherein

R_1 =  or 

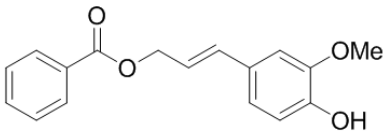
wherein

R_2, R_3, R_4, R_5, R_6 and R_7 are independently of each other H, OH or OCH_3.

2. The use of a composition according to claim 1, wherein each of R_5, R_6 and R_7 are independently of each other H, OH or OCH_3, and wherein preferably at most two of R_5, R_6 and R_7 are independently of each other OH or OCH_3.

3. The use of a composition according to any one of claims 1 to 2, wherein R_2, R_3 and R_4 are each H.

4. The use of a composition according to any one of claims 1 to 3, wherein said at least one compound of formula (I) or formula (II) is selected from compound **1** (coniferyl benzoate), **2** (p-coumaryl cinnamate), **3** (benzyl cinnamate), **4** (benzyl benzoate) and **5** (cinnamyl cinnamate).



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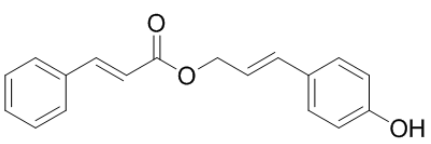

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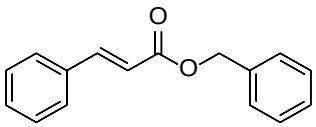

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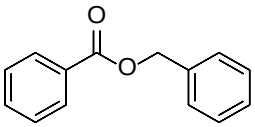

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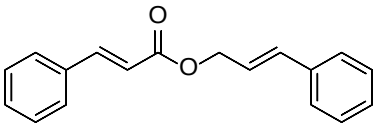


5

5. The use of a composition according to any one of the claims 1 to 4, wherein said composition comprises at least one, preferably exactly one, plant extract, and wherein said plant extract comprises said at least one compound of formula (I) or formula (II).

6. The use of a composition according to claim 5, wherein said at least one plant extract isan extract from a plant of the family of *Styracaceae.*

7. The use of a composition according to claim 5, wherein said at least one plant extract is an extract from a plant of the genus *Myroxylon.*

8. The use of a composition according to any one of the claims 5 to 7, wherein said at least one plant extract is an extract from a plant of the genus *Styrax* or a plant of the genus *Myroxylon*, and wherein said plant of the genus *Styrax* is selected from the species *Styrax tonkinensis*, *Styrax benzoin*, *Styrax paralleloneurum*, *Styrax hypoglauca* and *Styrax cascarifolia* or a subspecies or variety thereof, and wherein said plant of the genus *Myroxylon* is selected from the species *Myroxylon balsamum* and *Myroxylon peruiferum.*

9. The use of a composition according to any one of the claims 5 to 8, wherein said at least one plant extract is an extract of a resin of said plant, wherein preferably said at least one plant extract is an extract of a resin of Siam benzoin or Sumatra benzoin or Balsam of Peru.

10. The use of a composition according to any one of the preceding claims for controlling a plant fungal pathogen, wherein preferably said plant fungal pathogen is selected from (i) oomycetes, (ii) ascomycetes and (iii) basidiomycetes, wherein further preferably said plant fungal pathogen is selected from (i) oomycetes, (ii) ascomycetes and (iii)

basidiomycetes, and wherein said (i) oomycetes are selected from the genera *Hyaloperonospora, Peronospora, Plasmopara, Bremia, Pseudoperonospora* and *Phytophthora*; and wherein said (ii) ascomycetes are selected from the genera *Alternaria, Guignardia, Venturia, Oidium, Erysiphe, Sphaeroteca, Leveillula, Podosphaeria, Marssonina, Taphrina, Septoria, Sclerotinia, Pseudocercosporella, Botrytis, Phomopsis, Pyrenospora*; *Helminthosporium, Drechslera* and *Pyrenophora*; and wherein said (iii) basidiomycetes are selected from the genera *Puccinia, Phacopsora*, and *Rhizoctonia*.

11. The use of a composition according to any one of the preceding claims for controlling a fungal infection of a plant, plant propagation material or soil, preferably of a plant or plant propagation material, and again further preferably of a plant, wherein again further preferably said fungal infection is a fungal infection of a crop or a forestry plant, and wherein again further preferably said fungal infection is a fungal infection of a crop.

12. The use of a composition according to claim 11, wherein said fungal infection is a fungal infection of a crop selected from a fruit crop or a vegetable, wherein preferably said fruit crop is a grapevine plant or an apple tree and wherein preferably said vegetable is a tomato plant.

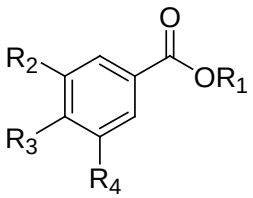
13. The use of a composition according to any one of claims 10 to 14, wherein said controlling said plant fungal pathogen or said controlling said fungal infection of said plant, plant propagation material or soil, comprises applying an effective amount of said composition to said plant, plant propagation material or soil, preferably to said plant or plant propagation material, and further preferably to said plant, wherein preferably said effective amount of said composition applied to said plant, plant propagation material or soil, preferably to said plant or plant propagation material, and further preferably to said plant, is an amount of said composition sufficient to provide a concentration of said at least one compound of formula (I) or formula (II) of 0.02% or a concentration of 0.02%, of the sum of all of said at least one compound of formula (I) and formula (II), or to provide a concentration of said at least one plant extract, preferably said extract of a resin of said plant, of 0.05%

14. The use of a composition according to any one of claims 1 to 13, wherein said composition is adapted as a formulation, wherein preferably said formulation is selected from a wettable powder, an emulsifiable concentrate, a water-dispersible granule, an emulsifiable granule, a microemulsion concentrate, an oil-in-water (EW) or water-in-oil (WO) emulsion, a suspo-emulsion and a capsule suspension, and wherein further preferably said formulation is selected from a wettable powder, an emulsifiable concentrate, a water-dispersible granule or an emulsifiable granule.

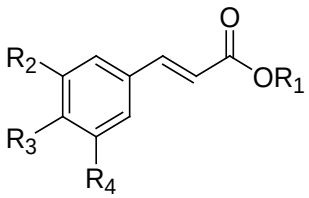
15. Use of a composition as a fungicide, wherein said composition comprises at least one plant extract, wherein said at least one plant extract is an extract from a plant of the family of *Styracaceae* or a plant of the genus *Myroxylon*, wherein preferably said at least one plant extract is an extract of *Styrax tonkinensis*, *Styrax benzoin*, *Styrax paralleloneurum* or *Myroxylon balsamum*, and wherein further preferably said at least one plant extract is an extract of a resin of Siam benzoin or Sumatra benzoin or Balsam of Peru.

ABSTRACT

The present invention relates to the use of a composition as a fungicide, wherein said composition comprises at least one compound of formula (I) or formula (II)

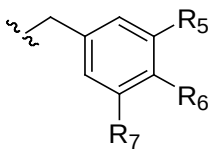


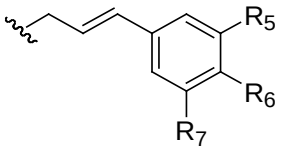
(I)



(II)

wherein

R_1 = 

or 

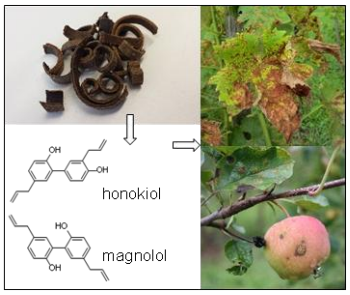
wherein

R_2, R_3, R_4, R_5, R_6 and R_7 are independently of each other H, OH or OCH_3.

3.3. Efficacy of a *Magnolia officinalis* Bark Extract against Grapevine Downy Mildew and Apple Scab under Controlled and Field Conditions

Barbara Thuerig, Justine Ramseyer, Matthias Hamburger, Mathias Ludwig, Thomas Oberhänsli, Olivier Potterat, Hans-Jakob Schärer, and Lucius Tamm

Crop Protection, in press



Magnolia officinalis (Magnoliaceae) bark extracted with ethyl acetate showed promising antifungal activity *in vitro*. On seedlings, *M. officinalis* bark extract showed a mean efficacy of 97% (*Plasmopara viticola*) and 93% (*Venturia inaequalis*) at 1 mg/mL. Efficacy against *Phytophthora infestans* was comparatively low (52% at 1 mg/mL). Magnolol and honokiol were identified as the main active compounds. Under field conditions, efficacies up to 71% were reached at 1-2 mg/mL against grapevine downy mildew, whereas activity against apple scab could not be confirmed.

Extraction of plant material, HPLC-microfractionation, quantifications, preparation of Figures 2 and 3, writing the drafts of Chapter 2.1. and a part of Chapter 3.2. were my contributions to this publication.

Justine Fabienne Ramseyer

Efficacy of a *Magnolia officinalis* bark extract against grapevine downy mildew and apple scab under controlled and field conditions

Barbara Thuerig[a*], Justine Ramseyer[b], Matthias Hamburger[b], Mathias Ludwig[a], Thomas Oberhänsli[a], Olivier Potterat[b], Hans-Jakob Schärer[a], and Lucius Tamm[a]

[a]Research Institute of Organic Agriculture FiBL, Ackerstrasse 113, CH-5070 Frick, Switzerland

[b]Division of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel, Klingelbergstrasse 50, CH-4056 Basel, Switzerland

[*]Corresponding author:

E-mail addresses: barbara.thuerig@fibl.org; justine.ramseyer@unibas.ch; matthias.hamburger@unibas.ch; mathias.ludwig@fibl.org; thomas.oberhaensli@fibl.org; olivier.potterat@unibas.ch; hans-jakob.schaerer@fibl.org; lucius.tamm@fibl.org

Declaration of interests: none

Highlights

- A *Magnolia officinalis* extract was efficient against three important plant pathogens
- Pathogens included *Plasmopara viticola*, *Venturia inaequalis* and *Phytophthora infestans*
- Efficacy was shown under controlled and field conditions
- Magnolol and honokiol were identified as the main active compounds

Abstract

In organic agriculture, the control of several diseases is largely depending on copper fungicides. Yet, copper can accumulate in the soil if the annual input exceeds annual uptake by plants, which can have a negative impact on soil fertility. Its use should thus be avoided or reduced. The aim of the present study was to evaluate the efficacy of a bark extract of *Magnolia officinalis* Rehder and Wilson to control three pathogens including *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni (causing grapevine downy mildew), *Venturia inaequalis* (Cooke) G. Winter (causing apple scab), and *Phytophthora infestans* (Mont.) De Bary (causing potato and tomato late blight) under controlled and field conditions, and to identify compounds responsible for the antifungal activity of the extract. Under controlled conditions, *M. officinalis* bark extract showed a mean efficacy of 97% (*P. viticola*) and 93% (*V. inaequalis*) at 1 mg mL^{-1}, and EC_{50} between 0.14 and 0.20 mg mL^{-1}. Efficacy against *P. infestans* was comparatively low (52% at 1 mg mL^{-1}). Magnolol and honokiol were identified as the main active compounds, both with $EC_{50} \leq 0.08$ mg mL^{-1} against *P. viticola* and *V. inaequalis*. Under field conditions, preliminary formulations reached efficacies up to 71% at 1-2 mg plant extract mL^{-1} against grapevine downy mildew, whereas activity against apple scab could not be confirmed. *Magnolia officinalis* is a promising candidate for the development of a sustainable plant protection product against grapevine downy mildew due to a combination of good efficacy, high availability of the raw material at affordable prices, reasonable extraction efficiency, and expected low human toxicity due to its longstanding use in traditional Chinese medicine.

Keywords: plant extract, fungicide, botanical plant protection product, honokiol, magnolol, *Magnolia officinalis*

1. Introduction

Plant pathogens are a constant threat to crops and can seriously compromise yields. Besides indirect measures such as crop rotation, selection of resistant or tolerant varieties, and habitat management, direct plant protection by fungicides is often essential to avoid severe yield losses due to pathogen infections. However, there is growing demand to replace chemical fungicides by more sustainable alternatives due to concerns about their impact on human health and the environment (Bolognesi, 2003; Gilliom, 2007; Mullin et al., 2010; Schwarzenbach et al., 2010; Weisenburger, 1993). Copper-based fungicides have been widely used to control many devastating plant diseases, including fungal and bacterial leaf spots, blights, anthracnoses, downy mildews and cankers (Agrios, 2005). The use of copper-based fungicides has decreased in conventional agriculture with the introduction of synthetic pesticides, and in organic agriculture thanks to improved formulations and the implication of decision support systems. Yet, copper input often still exceeds its uptake by plants, resulting in accumulation in the soil, especially in the case of perennial high-value crops such as grapevine or apple trees, due to a combination of intensive spray programmes with no or limited crop rotation (Eijsackers et al., 2005). Natural products such as plant extracts might provide effective, sustainable, and environmentally-friendly alternatives (Isman, 2014; Seiber et al., 2014). The use of plant extracts as insecticides has quite a long tradition (Ntalli and Menkissoglu-Spiroudi, 2011). For example, extracts from *Tanacetum cinerariifolium* (containing pyrethrines) (Casida, 1980), *Azadirachta indica* (containing azadirachtine) (Schmutterer, 1990) or *Quassia amara* (containing quassin) (Mancebo et al., 2000) very efficiently control several insect pests. They are mainly used in high value crops, herbs and ornamentals due to their relatively high prize (Isman, 2008). In contrast, only few plant extracts can be used in Europe against plant diseases, e.g. fennel oil (against powdery mildews and rust), lecithine (against powdery mildews), coconut potassium soap (against rainspot disease of apples), laminarine (an algae-derived stimulator of natural defense mechanisms in plants), and an extract of *Equisetum arvense* (against various diseases)

(Expert Group for Technical Advice on Organic Production EGTOP, 2016; Yoon et al., 2013a).

To identify extracts with antimicrobial activity, we screened a library of more than 3000 extracts originating from approximately 800 plant and fungal species for activity against important plant pathogens of high value crops, including *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni (causing grapevine downy mildew), *Venturia inaequalis* (Cooke) G. Winter (causing apple scab) and *Phytophthora infestans* (Mont.) De Bary (causing tomato and potato late blight)(Thuerig et al., 2016). These plant pathogens can cause up to 100% yield losses in non-treated plants, and plant protection in organic production is largely depending on copper fungicides (Finckh et al., 2015). Among the extracts tested, an ethyl acetate extract of *Magnolia officinalis* Rehder and Wilson bark showed promising activity. *Magnolia officinalis* is a deciduous tree distributed throughout subtropical China at elevations between 300 and 2000 m above sea level, and growing in natural broadleaf forests and plantations (Tong et al., 2002; Xiao et al., 2012). *Magnolia officinalis* is a well-known Asian medicinal plant, and its stem bark has been traditionally used in China, Korea and Japan to treat gastrointestinal disorders, anxiety and allergic diseases (Lee et al., 2011). It contains alkaloids and phenolic compounds, with the neolignans magnolol and honokiol contributing to 40-90% of total polyphenols (Poivre and Duez, 2017; Tong et al., 2002). The bark contains up to 7% of the two compounds, depending on factors including origin and age of the tree (Tong et al., 2002). Chinese and European Pharmacopoeia require a minimal content of 2% of neolignans in the herbal drug (Council of Europe, 2013; CPC (Chinese Pharmacopoeia Commission), 2010). Numerous therapeutic properties have been described for magnolol and honokiol (Lee et al., 2011). Activity against human pathogens has been documented, including viruses (Lan et al., 2012), bacteria (e.g. acne causing bacteria (Park et al., 2004), periodontal pathogens (Ho et al., 2001), multi-drug resistant bacterial strains (Jacobo-Salcedo et al., 2011)), and fungi (Bang et al., 2000; Clark et al., 1981; Jacobo-Salcedo et al., 2011). *Magnolia* sp. extracts and isolated compounds (honokiol, 1-methoxyhonokiol, magnolol and eudesmone) reportedly showed *in vitro* activity against some wood-decay fungi

and plant pathogens (Choi et al., 2009; Mori et al., 1997a; Mori et al., 1997b). An *in vivo* effect against some plant diseases (rice blast, tomato late blight, wheat leaf rust, barley powdery mildew, red pepper anthracnose) was shown under controlled conditions (Choi et al., 2009), and a formulated powder from *M. officinalis* stem bark reduced rust diseases of *Perilla* and *Zoysia* grass under field conditions (Yoon et al., 2013b).

The aim of the present study was to evaluate the potential of a *M. officinalis* extract to protect grapevine plants, apple trees and tomatoes against *Plasmopara viticola*, *Venturia inaequalis* or *Phytophthora infestans* under controlled and field conditions, and to identify the active constituents of the extract.

2. Material and methods

2.1. Phytochemistry

2.1.1 Chemicals

Solvents and formic acid were obtained from Scharlau (Barcelona, Spain). For extraction, technical grade solvents were used after re-distillation. For high-performance liquid chromatography (HPLC), HPLC-grade solvents were employed. HPLC grade water was obtained from a MilliQ water purification system (Merck Millipore, Darmstadt, Germany). Magnolol and honokiol references were purchased from Tokyo Chemical Industry (Tokyo, Japan).

2.1.2 Plant material

The bark of *Magnolia officinalis* var. *biloba* Rehder and Wilson was purchased from Peter Weinfurth, Bochum, Germany. The plant material was imported from China. A voucher specimen (Nr 216) is kept at the Division of Pharmaceutical Biology, University of Basel, Switzerland.

2.1.3 Extraction

The extract used for all controlled conditions bioassays and for field trials in 2014 was obtained as follows. The bark of *M. officinalis* was milled with a grinder SM 100 (Retsch,

125 Haan, Germany). The powder (14 kg) was divided into three portions and mixed with sea
126 sand (1:1). Each portion was then packed into a column, and percolated for 3 days with
127 approx. 32 L of ethyl acetate. After evaporation under reduced pressure, a total amount of
128 1053 g of extract was obtained (yield 7.5%) (Extract 1).

129 For field trials in 2015, 20 kg of *M. officinalis* finely sliced bark was macerated with 120 L of
130 ethyl acetate for 24 h. After filtration over a 50 µm polyester fleece the extract was
131 concentrated under reduced pressure to a liquid extract (3.0 kg) containing 32.6% dry matter
132 (yield 4.9%) (Extract 2).

133 2.1.4 HPLC microfractionation

134 Microfractionation was performed by semi-preparative HPLC on an Agilent 1100 Series with
135 a PDA detector (Santa Clara, CA, USA) connected to a FC204 fraction collector (Gilson,
136 Middleton, WI, USA). Separations were carried out at 25°C on a SunFire™ Prep C_{18} column
137 (5 µm, 150 x 10 mm i.d., Waters, Milford, MA, USA) equipped with a guard column (10 x 10
138 mm i.d.). The mobile phase consisted of water with 0.1% formic acid (Solvent A) and
139 acetonitrile with 0.1% formic acid (Solvent B). A gradient of 5 to 100% B in 30 min was used,
140 followed by 100% B for 5 min. The flow rate was 4 mL min^{-1}. The extract was dissolved in
141 DMSO at a concentration of 50 mg mL^{-1}, centrifuged, and filtered. Two injections of 200 µL
142 each were performed (20 mg of extract in total). Microfractions were collected every 90 sec
143 from 1 to 34 min (22 fractions for each run). After removal of the solvent in a Genevac EZ-2
144 evaporator (Stone Ridge, NY, USA), the fractions were redissolved in 300 µL of methanol.
145 The corresponding fractions obtained from the two separations were combined and dried.

146 2.1.5 Quantification of the active constituents

147 Analyses were performed in triplicate on an HPLC Agilent 1100 Series with a PDA detector
148 (Santa Clara, CA, USA). Separation was carried out at 25°C on a SunFire™ C_{18} (3.5 µm, 150
149 x 3.0 mm i.d.) column equipped with a guard column (10 mm x 3.0 mm i.d.). The mobile
150 phase consisted of water with 0.1% formic acid (Solvent A) and acetonitrile with 0.1% formic
151 acid (Solvent B). The flow rate was 0.4 mL/min. The following gradient was used: 50 to 100%

B in 20 min, followed by 100% B for 5 min. Samples were dissolved in DMSO at a concentration of 1 mg mL^{-1} for the extract, and 32-250 µg mL^{-1} for magnolol and honokiol. The injection volume was 10 µL. Detection was at 290 nm. Calibration curves were used to determine the concentration of the compounds in the extract: magnolol: 38.708x + 29.247 (r^2 = 0.9998); honokiol: 38.141x + 25.292 (r^2 = 0.9999).

2.2 Pathogens

Phytophthora infestans (Mont.) de Bary was cultivated on V8 agar (200 mL L^{-1} Campbell's V8 or "Biotta® Gemüsecocktail" (vegetable juice) (Biotta AG, Tägerwilen, Switzerland), 3 g L^{-1} $CaCO_3$, 1.5% Agar, pH 6.3) at 20°C in the dark. *Venturia inaequalis* Cooke (Wint.) was maintained on apple (*Malus domestica* Borkh.) seedlings cv. 'Jonagold' as described below. Leaves with sporulating lesions were dried at room temperature before storing them in glass vessels at 4°C in the dark. *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni was maintained on grapevine (*Vitis vinifera* L.) seedlings cv. 'Chasselas' by weekly re-inoculation (described below). Sporangia suspensions of *P. viticola* (1.8-2.5 x 10^5 sporangia mL^{-1}) and conidia suspensions of *V. inaequalis* (1.5-2.0 x 10^5 conidia mL^{-1}) were prepared by washing fresh (*P. viticola*) or dry (*V. inaequalis*) sporulating leaves with demineralized water. Sporangia suspensions of *P. infestans* (1.2-1.5 x 10^5 sporangia mL^{-1}) were prepared by placing mycelium harvested from 10-14 d old cultures into demineralized water and vigorous shaking. Suspensions were filtered over a cheese cloth, and sporangia concentrations assessed using a Thoma cell counting chamber prior to adjustment to desired concentrations.

2.3 In vitro bioassays

2.3.1 General procedures

All *in vitro* experiments were performed in 96-well plates. Media appropriate for each pathogen were used, namely mineral water ('Evian') for *P. viticola,* demineralized water for *V. inaequalis*, and demineralized water containing 1 mL L^{-1} V8-medium (200 mL L^{-1} Campbell's V8, 3 g L^{-1} $CaCO_3$, pH 6.3) for *P. infestans.* Each test plate contained at least 16 non-treated

control wells. The effect of the solvent (DMSO) alone was tested in at least eight replicates in three concentrations per experimental set (profiling microfractions) or in one dilution series (MIC_{100}).

Sporangia suspensions of *P. viticola* (1.8-2.5 x 10^5 sporangia mL^{-1}) and *P. infestans* (1.2-1.5 x 10^5 sporangia mL^{-1}) and conidia suspensions of *V. inaequalis* (1.5-2.0 x 10^5 conidia mL^{-1}) were prepared as described above.

Inhibitory activity was assessed 2-3 h (*P. viticola*), one day (*P. infestans*), or two days (*V. inaequalis*) after set-up of the experiment. All assessments were made using a binocular at 50 to 100-fold magnification.

2.3.2 Testing of microfractions

To determine activity of microfractions against *P. viticola*, *V. inaequalis*, and *P. infestans*, each of the 22 fractions were re-dissolved in 70 µL DMSO. 6 µL of each fraction were then added to 96-well plates containing 94 µL of the medium appropriate for each pathogen. They were then serially diluted in the test plate 1:10 and 1:100 by adding 10 µL of the next higher concentration to 90 µL of the appropriate test medium, 10 µL of the lowest concentration being discarded, such that each well contained 90 µL. Then, 20 µL of a continuously stirred pathogen suspension were added to each well.

Degrees of inhibition were assigned to one of three inhibition levels, with ‘level 0’ = similar to water control, ‘level 1’ = distinct reduction in germination rate and/or length of germ tubes (*V. inaequalis/P. infestans*) or reduced number and/or activity of zoospores (*P. viticola*), and ‘level 2’ = no germination, germ tubes shorter than half a sporangium (*V. inaequalis*) or conidium (*P. infestans*), or no zoospores germinated or all zoospores dead (*P. viticola*). The inhibition levels of the two lower tested concentrations were summed, resulting in values between 0 (no inhibition at the second highest tested concentration) and 4 (complete inhibition at lowest tested concentration).

204 2.3.3 Determination of minimal inhibitory concentrations (MIC_{100})

205 To determine the concentrations needed to completely inhibit germination of spores or
206 activity of zoospores (MIC_{100}), test substances (*M. officinalis* extract, magnolol, honokiol)
207 were dissolved in DMSO at concentrations of 10 or 5 mg mL^{-1}. They were then serially diluted
208 1:1 in water down to 0.02 mg mL^{-1} (10 concentrations). 6 µL of each test substance and
209 dilution were added to a well containing 94 µL of the appropriate medium, and 20 µL of
210 pathogen suspension was finally added. MIC_{100} is defined as the lowest concentration
211 sufficient for complete inhibition (inhibition 'level 2').

212 *2.4 Plant-pathogen bioassays*

213 2.4.1 Test products

214 For most controlled conditions bioassays, *M. officinalis* extract was dissolved in DMSO,
215 isopropylidenglycerol or EtOH at concentrations of 50 or 100 mg mL^{-1}, and then diluted with
216 water to concentrations between 4 and 0.1 mg mL^{-1}. For field experiments in 2014, *M.*
217 *officinalis* extract (Extract 1) was dissolved in isopropylidenglycerol at 100 mg mL^{-1} before
218 diluting into water.

219 For field experiments in 2015 and some controlled conditions bioassays, *M. officinalis* extract
220 (Extract 2) was used in formulations to improve handling and application. A wettable powder
221 (WP) formulation was developed containing 25% *M. officinalis* extract, 41% synthetic
222 amorphous silica and 9% wetting/dispersing agents and emulsifiers. An emulsifiable
223 concentrate (EC) formulation was developed containing 25% *M. officinalis* extract, 63%
224 solvents, and 12% emulsifiers.

225 2.4.2 Controlled conditions bioassays

226 Plant-pathogen bioassays were carried out under controlled conditions in experimental
227 facilities (greenhouse and growth chambers). Small grapevine (*cv.* 'Chasselas') or apple (*cv.*
228 'Jonagold') seedlings were transplanted to individual pots (0.275 L) containing a standard
229 substrate ('Einheitserde Typ 0', Gebr. Patzer GmbH & Co. KG, Sinntal-Jossa, Germany)
230 previously amended with 3 g L^{-1} of a mineral fertilizer (Tardit 3M, Hauert Günther
231 Düngerwerke GmbH, Erlangen, Germany). Plants were grown in the greenhouse at a

minimal temperature of 18°C under natural light. The photoperiod was extended with mercury high-pressure lamps resulting in a a light period of 16 hours. Plants were used for bioassays when they had 3-4 fully developed leaves (2-3 weeks after transplanting).

Each experimental set included a non-treated non-inoculated control, a water-treated inoculated control, a standard treatment ('Kocide Opti' containing 30% Cu^{2+} in the form of $Cu(OH)_2$, DuPont de Nemours, Wilmington, DE, USA) at two concentrations (0.3 g L^{-1} (concentration with expected excellent efficacy under field conditions) and 0.03 g L^{-1} of Cu^{2+}), and at least 12 test treatments. All experiments included six replicate plants per treatment. Plants were sprayed with the test products using an air-assisted hand sprayer (DeVilbiss® Compact MINI HVLP Touch-Up Spray Gun) or an automatic spray cabinet until leaves (adaxial and abaxial side) were completely covered with a dense layer of small droplets. Plants were subsequently left to dry at room temperature before inoculation.

Plasmopara viticola, *P. infestans* (both 5×10^4 sporangia mL^{-1}) and *V. inaequalis* (7×10^4 conidia mL^{-1}) were prepared as described above. Plants were spray-incoculated using an air-assisted hand sprayer on the abaxial (*P. viticola*) or the adaxial (*V. inaequalis, P. infestans*) leaf side. Inoculated plants were subsequently incubated at 20-21°C and 95-100% of relative humidity (RH) in the light for 24 h. Then, plants were maintained at 20°C, 60-80% RH, and a 16/8-h day/night light regime. For grapevine bioassays, 5 to 6 d after inoculation, plants were incubated over night in the dark at 20°C and 95-100% RH to promote sporulation.

Disease incidence (percentage of leaves with disease symptoms) and disease severity (percentage of leaf area covered by lesions) were assessed 5 d (*P. infestans*), 6 to 7 d after inoculation (*P. viticola*), or 10 to 12 d after inoculation (*V. inaequalis*). All disease assessments were made using continuous values of percentage: EPPO (European and Mediterranean Plant Protection Organization) standard scale (EPPO, 2001) for grapevine, scales suggested by Croxall et al. (1952) and Tehon and Stout (1930) for apple, and scale suggested by Corrêa et al. (2009) for tomato.

2.4.3 Field Trials

General procedures

Efficacy of *M. officinalis* extract against grapevine downy mildew (*P. viticola*), powdery mildew (*Erysiphe necator* Schwein.), and apple scab (*V. inaequalis*) was tested under field conditions (only natural infections, no artificial inoculation). The experiments were conducted following EPPO guidelines (PP 031/1 *P. viticola* (EPPO, 2001), PP 005/3 *V. inaequalis* (EPPO, 1990), PP 152/4 Design and analyses of efficacy evaluation trials (EPPO, 2012b), PP 181/4 Conduct and reporting of efficacy trials-GEP (good experimental practice) (EPPO, 2012a), PP 135/3 Phytotoxicity assessment (EPPO, 2014)). An untreated control served as a reference for natural development of disease epidemic. Phenological stages at disease assessments were recorded following the BBCH (Biologische Bundesantalt, Bundessortenamt and Chemical Industry) scale (Lorenz et al., 1995; Meier et al., 2009).

Experimental sites and design

The experiment was carried out in the screening-vineyard and -orchard in Frick, Switzerland, at 385 meters a.s.l, on a clay loam. The coordinates of the experimental plots are: 47°31'4'' N 08°01'33'' E. Average annual rainfall is 1138 mm (mean 2005-2014).

The experimental vineyard was established in 1997 and consists of 576 plants of the susceptible grapevine varieties 'Müller-Thurgau' ('Riesling x Sylvaner') and 'Chasselas' ('Gutedel') (288 plants per variety). Plant distance between rows is 2 m, within rows 1.1 m (4545 plants/ha). The experiment was set up in a complete randomized block design, with twelve treatments arranged in four replicates of six plants for both grapevine varieties. Due to the age of the vineyard and its previous use in trials, there are some plants of low quality. This resulted in some replicates having less than twelve plants.

The experimental orchard was established in 2007 and consists of 120 plants of the apple scab susceptible apple variety 'Pinova' grafted on rootstock M9. Plant distance between rows is 3 m, within rows 1 m. Six different treatments are arranged in a complete randomized block design, with four replicates consisting of five plants per replicate.

Experimental plots are maintained according to guidelines for organic agriculture. Weather data were recorded throughout the season with a Campbell weather station close to the experimental plot.

288 References, test products and applications

289 References: In grapevine, copper in the form of copper hydroxide (Kocide® Opti) was used
290 as a reference at 0.03 g Cu^{2+} L^{-1}. As a reference in apple trees, and as a second reference in
291 grapevine, a plant protection and application strategy recommended by the FiBL-advisory
292 service to Swiss organic grapevine and apple producers (‘organic reference’) was included.
293 In grapevine, ‘organic reference’(Häseli et al., 1999; Tamm et al., 2004) starts with the use of
294 Myco-Sin® (Andermatt Biocontrol, Grossdietwil, Switzerland; containing 65% acidified clay
295 minerals, 0.2% horsetail extract, concentration of formulation 0.8%) plus wettable sulphur
296 ‘Stulln’ (Andermatt Biocontrol, 80% sulphur, concentration of formulation 0.5%) in tank
297 mixture. Around bloom, depending on infection pressure and rainfall, copper (0.03 g Cu^{2+} L^{-1},
298 Kocide® Opti) was sprayed instead until the end of the season. The change to copper was
299 conducted on 5 July 2014 or 22 June 2015, after 8 (2014) or 6 (2015) copper-free treatments.
300 In apple, ‘organic reference’ uses a combination of preventive and curative (‘stop-
301 treatments’) treatments. Preventive treatments are applied before potential infection periods
302 (8 g L^{-1} Myco-Sin® plus 5 g L^{-1} wettable sulphur ‘Stulln’). ‘Stop-treatments’ (3 g L^{-1} Thiovit®
303 (Syngenta AG, Basel, Switzerland containing 80% wettable sulphur) plus 5 g L^{-1} Armicarb®
304 (Andermatt Biocontrol AG, Grossdietwil, Switzerland, containing 85% potassium
305 bicarbonate)) are applied as indicated by the Decision Support System RIMpro (see section
306 below ‘Application strategies’) during or after heavy infection periods based on potential
307 ascospore discharge to stop beginning infections (germination of spores and elongation of
308 germ tubes).

309 Test products: In 2014, *M. officinalis* extract (Extract 1) was tested in grapevine and apple
310 trees at 1 g plant extract L^{-1} spray broth. In 2015, the formulated *M. officinalis* extract (Extract
311 2) was tested at 2 g plant extract L^{-1} in grapevine (EC- and WP-formulation) and apple trees
312 (WP-formulation only). *Magnolia officinalis* extract was used for preventive as well as for
313 ‘stop-treatments’.

314 Application technique: Products in the grapevine trial were applied using two pressure based
315 and pressure tank supported spray systems (spray gun: GTi Pro light pressure, DeVillbiss,

316 USA; pressure tank: pressure feed cup KB-522-SS, DeVillbiss, Scottsdale, AZ, USA; 4 bar
317 spray pressure). The two spray systems were calibrated to dispense similar amounts of
318 product per unit of time. Plants were treated by spraying the product from above and from
319 below, which resulted in a homogeneous coating of the abaxial and adaxial leaf surface.
320 Products in the apple trial were applied using a motorized Honda type WJR 2525 knapsack
321 sprayer with a Yamaho-nozzle, type 20-10. Spray volume for 20 trees (four replicates with
322 five trees each) was 6.5 L in the beginning of the season, and 8.5 L when trees were fully
323 developed. In grapevine and apple trials, spray distribution was verified using water-sensitive
324 paper (Novartis, Basel, Switzerland).

325 Application strategies: Grapevine plants and apple trees were treated according to weather
326 conditions and risk for infection, calculated by the decision support systems ‘Vitimeteo’
327 (Agroscope, 2018) or ‘RIMpro’ (Trapman, 2018). In grapevine trials, treatments started 6 May
328 (2014) or 13 May (2015) and ended on 20 August 2014 or 21 August 2015. In both years, a
329 total of 16 treatments were performed in intervals of 3 to 10 days. In apple trials, treatments
330 with test products started 4 April 2014 or 10 April 2015, and the primary season (end of
331 ascospore discharge) ended 13 May 2014 or 12 June 2015. A total of 9 (2014) or 10 (2015)
332 treatments were performed during this period. In 2014, seven out of nine treatments were
333 preventive treatments, and two were curative ‘stop-treatments’ applied after heavy infection
334 periods, whereas all ten treatments were preventive in 2015. At the end of the primary
335 seasons, all plants in all treatments received a ‘stop-treatment’.

Disease assessments

Plasmopara viticola (downy mildew): Three (5, 19 and 27 August 2014) or four (26 June, 3 July, 23 July, 18 August 2015) disease assessments were carried out by scoring disease incidence (proportion of leaves with symptoms) and disease severity (percentage of diseased leaf area, continuous scale, EPPO guidelines (EPPO, 2001)) of *P. viticola* on leaves (100 leaves per plant or, if there were less leaves, all leaves). In 2014, overall leaf and grape-cluster area damaged by downy and powdery mildew was assessed on 9 September 2014 for each treatment replicate. In 2015, percentage diseased leaf-area infected by *P. viticola* was assessed on 24 July 2015 (assessment of all grapes per plant, all plants per variety and replication).

Venturia inaequalis (apple scab): Disease incidence and severity on leaves was assessed twice during the primary season (16 May and 5 June 2014, 18 May and 3 June 2015). At each disease assessment, 200 leaves (100 leaves from each side of the three middle trees) were scored on each treatment block containing five apple trees in a row. Disease incidence was calculated as the percentage of leaves showing any sign of infection. Severity was evaluated by estimating the surface of the infected area (continuous scale suggested by Croxall et al. (1952) and Tehon and Stout (1930)) and later multiplied with the disease incidence to get the infected leaf area per treatment replicate.

2.5 Calculations and statistics

To calculate means and confidence intervals of MIC_{100} values, data were $\log_2$-transformed. 95% confidence intervals were calculated from transformed data as $A \pm 1.96*B*n^{-0.5}$, with A = mean MIC_{100}, B = standard deviation MIC_{100} and n = number of experiments. Data were transformed back to the linear scale for presentation in tables.

Efficacies were calculated according to Abbott (1925) as $(1- (A*B^{-1})) * 100$. In controlled bioassays, A is disease severity/incidence on an individual plant and B mean disease severity/incidence of control plants. In field experiments, A is the mean disease severity/incidence of a treatment and B is the mean disease severity/incidence of the non-treated control.

EC_{50} values were calculated according to Alexander et al. (1999) as follows: $\mathrm{EC50} = \mathrm{ConcA} - \frac{(\mathrm{A}-50\%\,\mathrm{max\,response})\mathrm{x}(\mathrm{ConcA}-\mathrm{ConcB})}{\mathrm{A}-\mathrm{B}}$, with A and B the nearest actually recorded responses on either side of 50% the maximal response (A > 50%, B < 50%) and ConcA and ConcB the corresponding concentrations. EC_{50} were only calculated if maximal response was close to 100%. Area under disease progress curves (AUDPC) were calculated as $AUDPC = \sum_{i=1}^{n-1} \frac{y_i+y_{i+1}}{2} \times (t_{i+1} - t_i)$, where y_i is disease severity at the i^{th} observation, t_i is date at the i^{th} observation, and n is the total number of observations.

Severity and incidence data were arcsin-transformed before analysis, AUDPC log-transformed. Homogeneity of variances was checked using Levene's test. To check for a treatment effect in field trials, an analysis of variance with the factors variety, treatment (fixed factors) and block (random factor) and treatment X variety was performed. In case of non-significant interactions between treatment and variety, data of the two varieties were pooled. Otherwise, analysis was performed individually for each variety. Treatments were compared using a post-hoc Tukey-B test.

3. Results

3.1 Fungicidal activity of Magnolia officinalis *bark extract*

The ethyl acetate extract of *M. officinalis* showed strong antimicrobial activity *in vitro*, with minimal inhibitory concentrations (MIC_{100}) of 12 μg mL^{-1} against *P. viticola*, 32 μg mL^{-1} against *V. inaequalis*, and 74 μg mL^{-1} against *P. infestans* (Tab.1). Under controlled conditions, *M. officinalis* extract at a concentration of 1 mg mL^{-1} showed a mean efficacy of 97% ± 5% (mean ± SD of five independent experiments) on grapevine seedlings against downy mildew caused by *P. viticola* under high to very high disease pressure (Tab. 2). EC_{50} determined in a concentration-response experiment was 0.2 mg mL^{-1} (Fig. 1A). On apple seedlings, efficacy of *M. officinalis* extract at a concentration of 1 mg mL^{-1} against apple scab caused by *V. inaequalis* was 93% ± 5% (mean ± SD of four independent experiments) (Tab. 3), and the EC_{50} determined in a concentration-response experiment was 0.14 mg mL^{-1} (Fig.

1B). On tomato seedlings the efficacy of *M. officinalis* extract against *P. infestans* was 82% at 4 mg mL^{-1} (1 experiment), 66% ± 25% at 2 mg mL^{-1} (mean ± SD of 3 independent experiments), and 52% ± 22% at 1 mg mL^{-1} (mean ± SD of 3 independent experiments) (Tab. 4). EC_{50} determined in a concentration-response experiment was 0.5 mg mL^{-1} (Fig. 1C).

3.2 Identification of the active constituents in Magnolia officinalis *bark extract*

To identify the active constituents, a process referred to as HPLC-based activity profiling was applied (Potterat and Hamburger, 2014). When the bioactivity data and chromatographic trace were compared (Fig. 2), the activity could be mainly assigned to Fractions 14 to 16 eluting between 20.5 and 25.0 min. These fractions contained two major peaks which were identified by HPLC-ESIMS analysis and comparison with reference substances as honokiol (*m/z* 265.2, $[M-H]^-$) (**1**) and magnolol (*m/z* 265.2, $[M-H]^-$) (**2**) (Fig 3)(Li et al., 1983). The extracts used in this study contained 13.3% magnolol and 4.3% honokiol (Extract 1), or 18.8% magnolol and 11.5% honokiol (Extract 2), as determined by HPLC-UV analysis.

In vitro, MIC_{100} of magnolol was between 3 (*P. viticola*) and 11 (*P. infestans*) μg mL^{-1}, and MIC_{100} of honokiol was between 1 (*P. viticola*) and 5 (*P. infestans*) μg mL^{-1} (Tab. 1). Under controlled conditions, 0.5 mg mL^{-1} and 0.25 mg mL^{-1} magnolol were sufficient to reach efficacies ≥ 90% against *P. viticola* and *V. inaequalis* respectively, whereas a maximal efficacy of 87% against *P. infestans* was reached at this concentration (Fig. 1). For honokiol, 0.065 mg mL^{-1} were sufficient to reach efficacies ≥ 90% against *P. viticola* and *V. inaequalis*, whereas a maximal efficacy of 54% against *P. infestans* was reached at 0.5 mg mL^{-1}, the highest tested concentration (Fig. 1).

3.3 Efficacy of Magnolia officinalis bark extract against grapevine downy mildew and apple scab under field conditions

3.3.1 Development of epidemics and efficacy of reference products

In March and April 2014, temperature was above the long-time average, followed by temperatures below the long-time average in May, and above the average until end of June,

417 with rainfall below the average for this period. The warm and dry weather conditions from
418 mid-May until end of June resulted in relatively low grapevine downy mildew disease
419 pressure in the primary infection period, whereas the development of powdery mildew
420 caused by *Erysiphe necator* was favoured. In August 2014, downy mildew developed rapidly
421 such that by the end of August, downy mildew disease incidence was close to 100% (data
422 not shown) and severity around 50% (Fig. 4A). For *V. inaequalis*, five primary ascospore
423 discharges with high infection rate were predicted by the RIMpro Decision Support System
424 from end of March to end of May 2014. The first ascospore discharge occurred on 22 March
425 2014, the last on 16 May 2014. First apple scab lesions were registered on 1 May 2014. At
426 the end of the primary season 5 June 2014, disease incidence (34%, Fig. 5A) and severity
427 (5%, data not shown) in the non-treated control plants was moderate.
428 In 2015, spring and summer temperatures were high, with average temperatures above the
429 long-time average. Relatively low precipitations in March and April 2015 were followed by
430 heavy precipitations in the beginning of May, relatively wet conditions in June and very dry
431 conditions during July and August 2015. In 2015, the first visible downy mildew symptoms
432 appeared in the beginning of June, with the disease progressing quite rapidly till the end of
433 June. The warm and dry weather conditions during July and August slowed down the
434 infection progress, such that by the end of August 2015, disease incidence and severity in
435 the non-treated control was moderate, reaching 50% and 9%, respectively (Fig. 4B). For *V.*
436 *inaequalis*, the first ascospore discharge occurred on 28 March 2015, the last on 6 May
437 2015. Five primary ascospore discharges with high infection rate were predicted by the
438 RIMpro-model between end of March and beginning of May 2015. First apple scab lesions
439 were registered on 27 April 2015. Apple scab disease incidence (84%, Fig. 5B) and severity
440 (14%, data not shown) in the non-treated control plants was very high by the end of the
441 primary season (3 June 2015).
442 For grapevine downy mildew, the references (copper and Strategy) protected grapevine
443 leaves and fruit very efficiently from downy mildew (86-94% reduction of infected leaf area

444 (Fig. 4), 88-96% reduction of infected bunch area (data not shown)) in both seasons.
445 Powdery mildew was completely inhibited in the copper control (data not shown).
446 For apple scab, the organic fungicide reference showed high efficacy in 2014 (efficacy
447 incidence 91% (Fig. 5A), efficacy severity 98% (data not shown)), whereas efficacy was
448 relatively low in 2015 due to severe weather conditions (efficacy incidence 48% (Fig. 5B),
449 efficacy severity 73% (data not shown)).

450 3.3.2 Efficacy of *Magnolia officinalis* bark extract
451 In both years, *M. officinalis* extract significantly reduced downy mildew disease severity,
452 although to different degrees. In 2014, *M. officinalis* extract reduced the leaf area infected by
453 *P. viticola* by approx. 50% at the first two disease assessments (Fig. 4A). At the end of the
454 season (end of August), efficacy was 26%. In this trial, *M. officinalis* extract was used without
455 additives, and a brown sticky precipitate was observed in the spray equipment. In 2015, *M.*
456 *officinalis* extract was tested in two preliminary formulations, an emulsifiable concentrate
457 (EC) and a wettable powder (WP). These formulations significantly reduced leaf area
458 diseased by *P. viticola* by approx. 75% at the beginning of the season (end of June) (Fig.
459 4B). In July, efficacies of 50% and 65%, respectively, were observed for the two
460 formulations. At the end of the season (end of August), efficacies of 55% and 71% were
461 reached, with the efficacy of the WP formulation not significantly differing from the efficacy of
462 the reference organic fungicide.

463 When tested under field conditions in 2014 and 2015 on apple trees of the variety 'Pinova',
464 *M. officinalis* bark extract did not reduce apple scab caused by *V. inaequalis* (Fig. 5).

465 ## 4. Discussion

466 In the present study, we showed for the first time that a *M. officinalis* bark extract could
467 efficiently control grapevine downy mildew caused by *P. viticola* and apple scab caused by *V.*
468 *inaequalis* when tested under controlled conditions. The two neolignans honokiol and
469 magnolol were identified as the main active compounds. Concentrations needed to reach
470 high efficacies (≥ 93% efficacy at 1 mg extract mL^{-1}) were relatively low. This is one of

several prerequisites for an economically viable plant protection product based on plant extracts. Against *P. viticola*, some other plant extracts had been previously shown to reach comparable efficacies at similar concentrations (e.g. extracts of *Juncus effusus, Abies sibirica, Inula viscosa*, *Yucca schidigera, Melaleuca alternifolia,* and *Quillaja saponaria*) (Dagostin et al., 2011; Thuerig et al., 2016), whereas 20 to 100 times higher concentrations were required for other extracts (e.g. *Glycyrrhiza glabra*, *Salvia officinalis*, *Solidago virgaurea*, *Rheum rhabarbarum*) (Dagostin et al., 2011). Choi et al. (2009) found that, under controlled conditions, 3 mg mL^{-1} of an apolar extract from the bark of *Magnolia obovata* and *M. officinalis* reduced rice blast caused by *Magnaporthe grisea*, wheat leaf rust caused by *Puccinia recondita*, barley powdery mildew caused by *Erysiphe graminis* f. sp. *hordei*, red pepper anthracnose caused by *Colletotrichum coccodes*, and tomato late blight caused by *P. infestans* by ≥ 90%. In the present study, we could confirm the fungicidal activity of *M. officinalis* bark extract towards tomato late blight at similar concentrations. Yet, activity against *P. infestans* was lower than against *P. viticola* and *V. inaequalis*, and therefore no further studies on tomato or potato plants were performed.

The efficacy of *M. officinalis* bark against grapevine downy mildew was confirmed under field conditions. In our screening vineyard, efficacies up to 71% were reached with a preliminary formulation of the extract, even though disease pressure in this experimental vineyard was very severe due to highly infected non-treated control plants standing immediate vicinity to treated plants. Only few (*Melaleuca alternifolia*, *Salvia officinalis*, and *Larix decidua*) plant extracts previously tested in the same vineyard reached similar efficacies (Dagostin et al., 2011; Thuerig et al., 2018). While we could not confirm efficacy against apple scab under field conditions, a study by Yoon et al. with a preliminary formulation of powdered *M. officinalis* stem bark against rust of Perilla and Zoysiagrass showed that a significant effect under field conditions is possible (Yoon et al., 2013b).

M. officinalis bark extract fulfills several prerequisites for the development of a sustainable and profitable plant protection product. Stem bark of several *Magnolia* species including *M. officinalis* has been used for hundreds of years in traditional Chinese and Japanese

medicine. It is currently also used as a dietary supplement and cosmetic ingredient, and its toxicity for humans is considered low (e.g. reviewed by Poivre and Duez (2017)). The active compounds honokiol and magnolol are present in significant amounts in the bark (Tong et al., 2002), extraction efficacy was reasonable (around 7%), with high concentrations of the active compounds in the resulting extract (up to 30% in the present study). In the present as well as in earlier studies honokiol and magnolol showed high activity at low concentrations against a broad spectrum of pathogens known to cause significant losses in important crops (Choi et al., 2009; Mori et al., 1997a; Mori et al., 1997b). Furthermore, bark of *M. officinalis* is available in significant amounts at an affordable price. However, reported activity of magnolol against brine shrimp (*Artemia salina*) (Nitao et al., 1991), larvae of mosquito (*Aedes aegypti*) (Nitao et al., 1991), Palamedes swallowtail butterfly (*Papilio palamedes*) (Nitao et al., 1992) and some nematodes (Li et al., 2009) indicates that toxicology of an *M. officinalis* extract against non-target organisms should be assessed prior to further product development.

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References

Abbott, W.S., 1925. A method for computing the effectiveness of an insecticide. J. Econ. Entomol. 18, 265-267.

Agrios, G.N., 2005. Plant pathology, 5th ed. Elsevier Academic Press, Burlington.

Agroscope, 2018. Agrometeo. http://www.agrometeo.ch/ (accessed 13 June 2018)

Alexander, B., Browse, D.J., Reading, S.J., Benjamin, I.S., 1999. A simple and accurate mathematical method for calculation of the EC50. J. Pharmacol. Toxicol. Methods 41, 55-58.

Bang, K., Kim, Y., Min, B., Na, M., Rhee, Y., Lee, J., Bae, K., 2000. Antifungal activity of magnolol and honokiol. Arch. Pharm. Res. 23, 46-49.

Bolognesi, C., 2003. Genotoxicity of pesticides: a review of human biomonitoring studies. Mutat. Res. 543, 251-272.

Casida, J.E., 1980. Pyrethrum flowers and pyrethroid insecticides. Environ. Health Persp. 34, 189-202.

Choi, N.H., Choi, G.J., Min, B.S., Jang, K.S., Choi, Y.H., Kang, M.S., Park, M.S., Choi, J.E., Bae, B.K., Kim, J.C., 2009. Effects of neolignans from the stem bark of *Magnolia obovata* on plant pathogenic fungi. J. Appl. Microbiol. 106, 2057-2063.

Clark, A.M., El-Feraly, A.S., Li, W.-S., 1981. Antimicrobial activity of phenolic constituents of *Magnolia grandiflora* L. J. Pharm. Sci. 70, 951-952.

Corrêa, F., Bueno Filho, J., Carmo, M., 2009. Comparison of three diagrammatic keys for the quantification of late blight in tomato leaves. Plant Pathol. 58, 1128-1133.

Council of Europe, 2013. European Pharmacopoeia 8.1. Council of Europe, Strasbourg.

CPC (Chinese Pharmacopoeia Commission), 2010. Pharmacopoeia of the People's Republic of China, 9th ed. China Medical Science Press, Beijing, China.

Croxall, H., Gwynne, D., Jenkins, J., 1952. The rapid assessment of apple scab on leaves. Plant Pathol. 1, 39-41.

Dagostin, S., Schärer, H.-J., Pertot, I., Tamm, L., 2011. Are there alternatives to copper for controlling grapevine downy mildew in organic viticulture? Crop Prot. 30, 776-788.

Eijsackers, H., Beneke, P., Maboeta, M., Louw, J., Reinecke, A., 2005. The implications of copper fungicide usage in vineyards for earthworm activity and resulting sustainable soil quality. Ecotox. Environ. Safe. 62, 99-111.

EPPO, 1990. *Venturia inaequalis, Venturia pirina*. EPPO Bulletin 20, 425-433.

EPPO, 2001. *Plasmopara viticola*. EPPO Bulletin 31, 313-317.

EPPO, 2012a. Conduct and reporting of efficacy evaluation trials, including good experimental practice. EPPO Bulletin 42, 382-393.

EPPO, 2012b. Design and analysis of efficacy evaluation trials. EPPO Bulletin 42, 367-381.

EPPO, 2014. PP 1/135 (4) Phytotoxicity assessment. EPPO Bulletin 44, 265-273.

Expert Group for Technical Advice on Organic Production EGTOP, 2016. Final report on plant protection (III). European Commission - Directorate-Général for Agriculture and Rural Development.

Finckh, M.R., van Bruggen, A.H., Tamm, L., 2015. Plant diseases and their management in organic agriculture, 1st ed. APS Press, St. Paul, Minnesota.

Gilliom, R.J., 2007. Pesticides in US streams and groundwater. Environ. Sci. Technol. 41, 3408-3414.

Häseli, A., Tamm, L., Wyss, E., 1999. Krankheits- und Schädlingsregulierung im biologischen Rebbau. Research Institute of Organic Agriculture, Frick, Switzerland.

Ho, K.Y., Tsai, C.C., Chen, C.P., Huang, J.S., Lin, C.C., 2001. Antimicrobial activity of honokiol and magnolol isolated from *Magnolia officinalis*. Phytother. Res. 15, 139-141.

Isman, M., 2008. Problèmes et perspectives de commercialisation des insecticides d'origine botanique, In: Regnault-Roger, C., Phellogène, B.J.R., Vincent, C. (Eds.), Biopesticides d'origine végétale, 2nd ed. Lavoisier Editions TEC&DOC, Paris, pp. 465-476.

Isman, M.B., 2014. Botanical Insecticides: A Global Perspective, In: Gross, A., Coats, J.R., Duke, S.O. (Eds.), Biopesticides: State of the Art and Future Opportunities. American Chemical Society, pp. 21-30.

568 Jacobo-Salcedo, M.R., Gonzalez-Espindola, L.A., Alonso-Castro, A.J., Gonzalez-Martinez, M.R.,
569 Dominguez, F., Garcia-Carranca, A., 2011. Antimicrobial activity and cytotoxic effects of *Magnolia*
570 *dealbata* and its active compounds. Nat. Prod. Commun. 6, 1121-1124.
571 Lan, K.H., Wang, Y.W., Lee, W.P., Lan, K.L., Tseng, S.H., Hung, L.R., Yen, S.H., Lin, H.C., Lee, S.D., 2012.
572 Multiple effects of honokiol on the life cycle of hepatitis C virus. Liver Int. 32, 989-997.
573 Lee, Y.-J., Lee, Y.M., Lee, C.-K., Jung, J.K., Han, S.B., Hong, J.T., 2011. Therapeutic applications of
574 compounds in the Magnolia family. Pharmacol. Therapeut. 130, 157-176.
575 Li, A.J., Guo, X.F., Wang, X.M., Chen, C.B., Shi, Y.H., Sui, N.H., Du, J.Q., 1983. Determination of
576 phenolic substances in Hou Po by HPLC. Chin. J. Pharm. Anal. 3, 1-3.
577 Li, G.-H., Dang, L.-Z., Hong, L.-J., Zheng, L.-J., Liu, F.-F., Li, L., Liu, Y.-J., Zhang, K.-Q., 2009. Nematicidal
578 activity of honokiol and magnolol isolated from *Magnolia tripetala*. J. Phytopathol. 157, 390-392.
579 Lorenz, D., Eichhorn, K., Bleiholder, H., Klose, R., Meier, U., Weber, E., 1995. Growth stages of the
580 grapevine: Phenological growth stages of the grapevine (*Vitis vinifera* L. ssp. *vinifera*)—Codes and
581 descriptions according to the extended BBCH scale. Aust. J. Grape Wine R. 1, 100-103.
582 Mancebo, F., Hilje, L., Mora, G.A., Salazar, R., 2000. Antifeedant activity of *Quassia amara*
583 (Simaroubaceae) extracts on *Hypsipyla grandella* (Lepidoptera: Pyralidae) larvae. Crop Prot. 19, 301-
584 305.
585 Meier, U., Bleiholder, H., Buhr, L., Feller, C., Hack, H., Heß, M., Lancashire, P.D., Schnock, U., Stauß,
586 R., Van Den Boom, T., 2009. The BBCH system to coding the phenological growth stages of plants–
587 history and publications. Journal für Kulturpflanzen 61, 41-52.
588 Mori, M., Aoyama, M., Doi, S., 1997a. Antifungal constituents in the bark of *Magnolia obovata*
589 Thunb. Holz Roh Werkst. 55, 275-278.
590 Mori, M., Aoyama, M., Doi, S., Kanetoshi, A., Hayashi, T., 1997b. Antifungal activity of bark extracts of
591 deciduous trees. Holz Roh Werkst. 55, 130-132.
592 Mullin, C.A., Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., vanEngelsdorp, D., Pettis, J.S., 2010.
593 High levels of miticides and agrochemicals in North American apiaries: implications for honey bee
594 health. PLoS one 5, e9754.
595 Nitao, J., Johnson, K., Scriber, J.M., Nair, M., 1992. *Magnolia virginiana* neolignan compounds as
596 chemical barriers to swallowtail butterfly host use. J. Chem. Ecol. 18, 1661-1671.
597 Nitao, J.K., Nair, M.G., Thorogood, D.L., Johnson, K.S., Scriber, J.M., 1991. Bioactive neolignans from
598 the leaves of *Magnolia virginiana*. Phytochemistry 30, 2193-2195.
599 Ntalli, N.G., Menkissoglu-Spiroudi, U., 2011. Pesticides of botanical origin: a promising tool in plant
600 protection. INTECH Open Access Publisher.
601 Park, J., Lee, J., Jung, E., Park, Y., Kim, K., Park, B., Jung, K., Park, E., Kim, J., Park, D., 2004. *In vitro*
602 antibacterial and anti-inflammatory effects of honokiol and magnolol against *Propionibacterium* sp.
603 Eur. J. Pharmacol. 496, 189-195.
604 Poivre, M., Duez, P., 2017. Biological activity and toxicity of the Chinese herb *Magnolia officinalis*
605 Rehder & E. Wilson (Houpo) and its constituents. J. Zhejiang Univ. Sci. B. 18, 194-214.
606 Potterat, O., Hamburger, M., 2014. Combined use of extract libraries and HPLC-based activity
607 profiling for lead discovery: Potential, challenges, and practical considerations. Planta Med. 80, 1171-
608 1181.
609 Schmutterer, H., 1990. Properties and potential of natural pesticides from the neem tree,
610 *Azadirachta indica*. Annu. Rev. Entomol. 35, 271-297.
611 Schwarzenbach, R.P., Egli, T., Hofstetter, T.B., Von Gunten, U., Wehrli, B., 2010. Global water
612 pollution and human health. Annu. Rev. Environ. Resour. 35, 109-136.
613 Seiber, J.N., Coats, J., Duke, S.O., Gross, A.D., 2014. Biopesticides: State of the art and future
614 opportunities. J. Agric. Food Chem. 62, 11613-11619.
615 Tamm, L., Fuchs, J.G., Böger, N., Mühletaler, L., Amsler, T., Levite, D., Häseli, A., 2004. Properties of
616 acidified clay preparations: the Swiss experience, Symposium für ökologischen Weinbau. Intervitis
617 Stuttgart, pp. 27-36.
618 Tehon, L.R., Stout, G.L., 1930. Epidemic diseases of fruit trees in Illinois 1922-1928. Illinois Natural
619 History Survey Bulletin 18, 415-502.

620 Thuerig, B., James, E.E., Schärer, H.-J., Langat, M.K., Mulholland, D.A., Treutwein, J., Kleeberg, I.,
621 Ludwig, M., Jayarajah, P., Giovannini, O., Markellou, E., Tamm, L., 2018. Reducing copper use in the
622 environment: The use of larixol and larixyl acetate to treat downy mildew caused by *Plasmopara*
623 *viticola* in viticulture. Pest Manag. Sci. 74, 477-488.
624 Thuerig, B., Ramseyer, J., Hamburger, M., Oberhänsli, T., Potterat, O., Schärer, H.J., Tamm, L., 2016.
625 Efficacy of a *Juncus effusus* extract on grapevine and apple plants against *Plasmopara viticola* and
626 *Venturia inaequalis*, and identification of the major active constituent. Pest Manag. Sci. 72, 1718-
627 1726.
628 Tong, Z.-K., Zeng, Y.-R., Si, J.-P., 2002. Variation, heredity and selection of effective ingredients in
629 *Magnolia officinalis* of different provenances. J. Forestry Res. 13, 7-11.
630 Trapman, M., 2018. RIMpro. http://www.rimpro.eu/faces/venturia.xhtml?id=1YQiMPH (accessed 12
631 June 2018)
632 Weisenburger, D.D., 1993. Human health effects of agrichemical use. Hum. Pathol. 24, 571-576.
633 Xiao, S., Xu, Y., Zhiling, Y., 2012. Variation in seed and seedling traits among fifteen Chinese
634 provenances of *Magnolia officinalis*. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 40, 274-283.
635 Yoon, M.-Y., Cha, B., Kim, J.-C., 2013a. Recent trends in studies on botanical fungicides in agriculture.
636 Plant Pathology J. 29, 1-9.
637 Yoon, M.-Y., Choi, Y.H., Kang, M.S., Lee, J.H., Han, S.S., Myoung, I.S., Han, B.S., Choi, G.J., Jang, K.S.,
638 Kim, J.-C., 2013b. Disease control efficacy of the extract of *Magnolia officinalis* against Perilla and
639 Zoysiagrass rusts. Res. Plant Dis. 19, 45-48.

640

Figure 1. Concentration-response curves of a *Magnolia officinalis* bark extract (MO), honokiol, and magnolol on grapevine (A1), apple (B1), and tomato (C1) seedlings against *Plasmopara viticola*, *Venturia inaequalis* or *Phytophthora infestans,* respectively. Each experiment included two concentrations (300 and 30 μg mL-1) of a copper reference (Cu^{2+}) (A2, B2, C2). A3, B3, C3: Disease severity in the non-treated control. The figures show mean ± SD (n=6).

Figure 2. Activity profiling of *Magnolia officinalis* ethyl acetate extract against *Plasmopara viticola*, *Phytophthora infestans*, and *Venturia inaequalis*. Fractionation of a total of 20 mg of extract was performed by semi-preparative HPLC; detection at 254 nm. Fractions were collected every 90 sec (22 fractions). Activity of each fraction was assessed against *P. viticola, P. infestans*, and *V. inaequalis*. Inhibition levels of two concentrations were summed up, resulting in values between 0 (no inhibition at second highest tested concentration) and 4 (complete inhibition at lowest tested concentration).

Fig. 3. Chemical structure of the two active compounds honokiol (**1**) and magnolol (**2**).

Fig. 4. Disease severity (A1, B1) and AUDPC (A2, B2) of downy mildew caused by *Plasmopara viticola* under field conditions (natural infections) in 2014 (A) and 2015 (B) on leaves of grapevine plants. Plants were either left untreated (Control) or treated with 0.3 g L^{-1} Cu^{2+} ('copper'), a plant protection strategy recommended by the FiBL-advisory service to Swiss grapevine producers ('organic reference') or a *Magnolia officinalis* bark extract. In 2014, *M. officinalis* bark extract was tested non-formulated at 2 g L^{-1} ('MO'), in 2015 it was tested as a wettable powder ('MO WP') and an emulsifiable concentrate ('MO EC') formulation at 2 g plant extract L^{-1}. The experiment was set up in a complete randomized block design with four replicates, each consisting of six grapevine plants *cv*. 'Müller-Thurgau'

667 and six plants *cv*. ‘Chasselas’. The figures show means of four treatment replicates. Different
668 lower case letters indicate significant differences between treatments at an individual date
669 (ANOVA on arcsin-transformed data followed by a post-hoc Tukey-B test to compare
670 treatments). The phenological stages (according to BBCH) are indicated.

671

672 **Fig. 5.** Disease incidence of apple scab caused by *Venturia inaequalis* under field conditions
673 in 2014 (A) and 2015 (B) on leaves of apple plants (*Malus domestica*) (natural infection).
674 Plants were either left untreated (Control), or plants were treated with a plant protection
675 strategy recommended by the FiBL-advisory service to Swiss organic apple producers
676 (‘organic reference’, Myco-Sin plus Sulphur, Armicarb), or a *Magnolia officinalis* bark extract.
677 In 2014, *M. officinalis* bark extract was tested non-formulated at 1-2 g L^{-1} (‘MO’). In 2015, *M.*
678 *officinalis* bark extract was tested in a wettable powder formulation (‘MO WP’) at 2 g L^{-1}. The
679 experiment was set up in a complete randomized block design with four replicates, each
680 consisting of five apple trees *cv.* ‘Pinova’. The figures show means ± SD of four treatment
681 replicates. Different lower case letters indicate significant differences between treatments at
682 an individual date (ANOVA on arcsin-transformed data followed by a post-hoc Tukey-B test
683 to compare treatments). The phenological stages (according to BBCH) are indicated.

684

685

Figure1

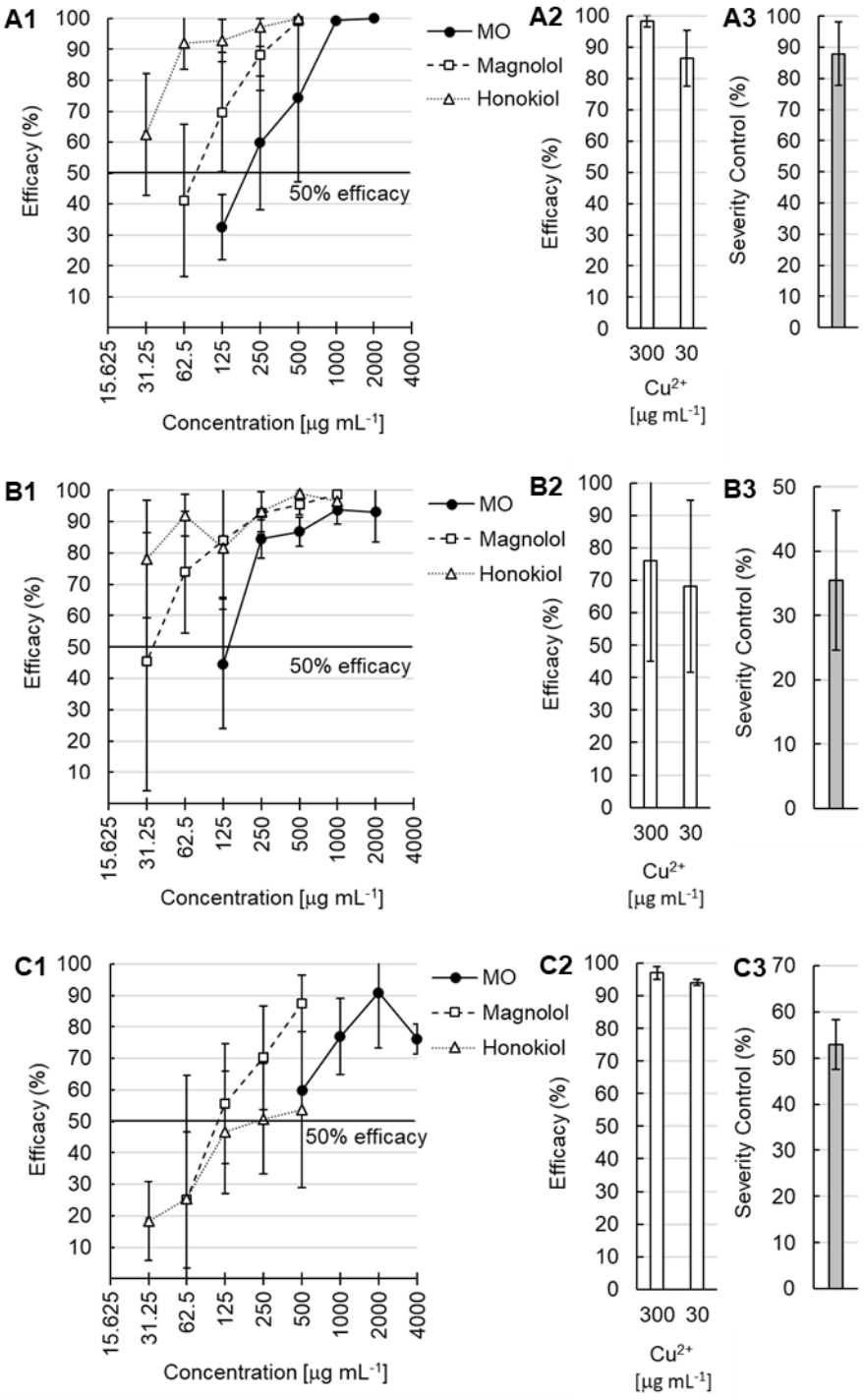


Fig. 1

Figure2

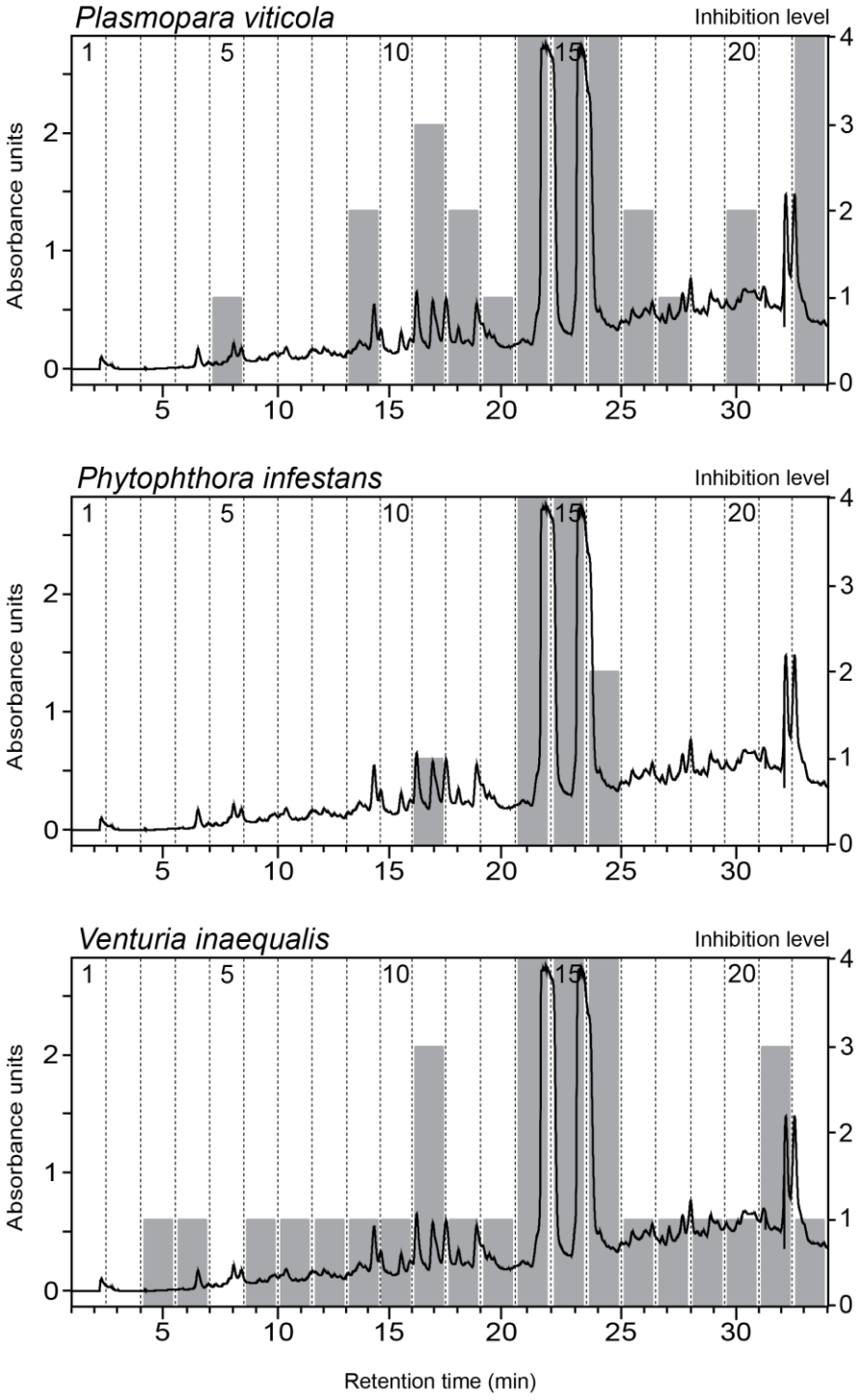
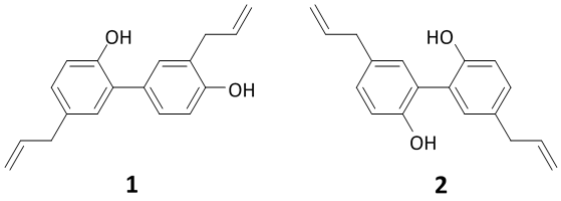


Fig. 2

Figure3



1 **2**

Fig. 3

Figure4

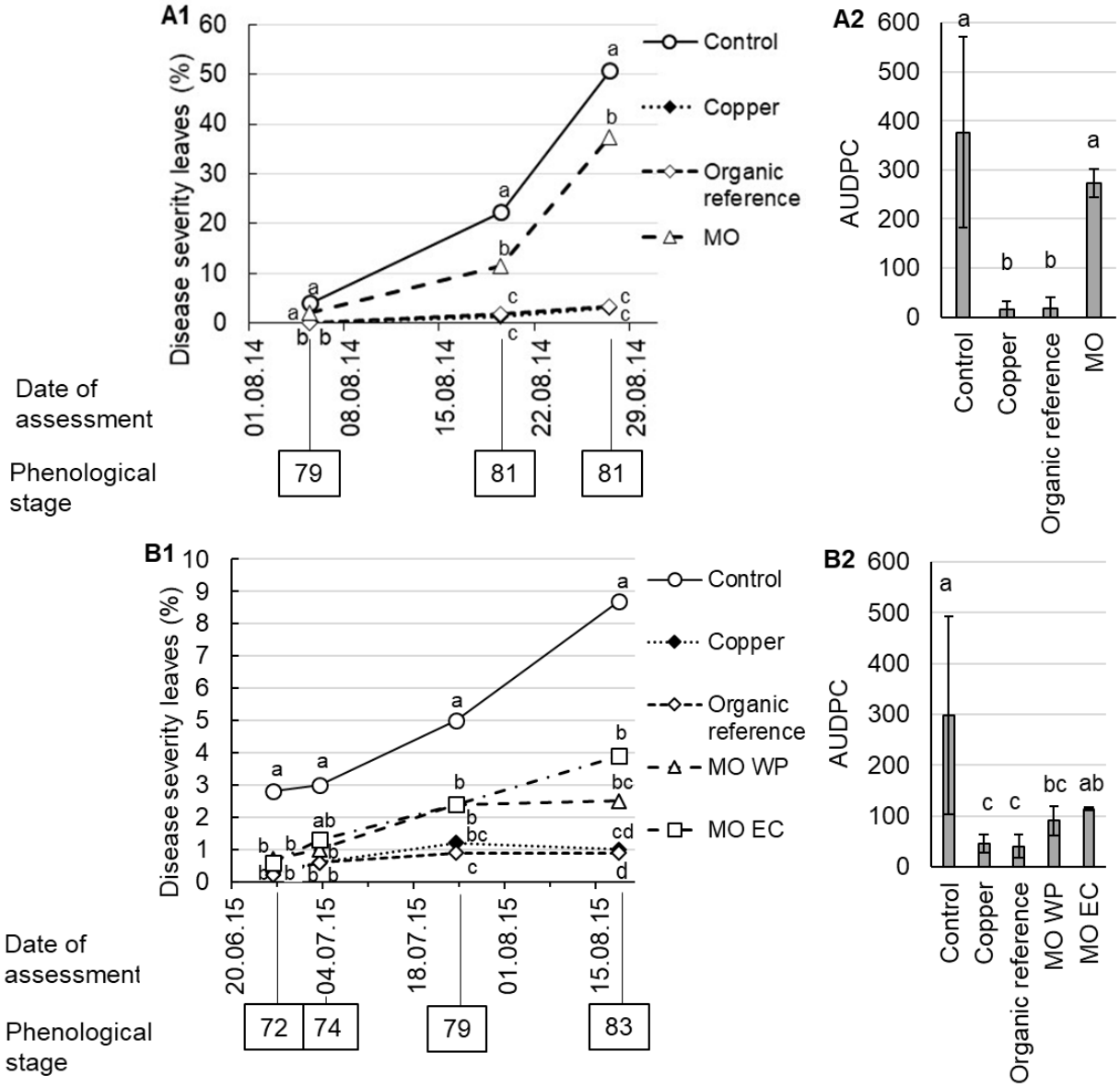


Fig. 4

Figure5

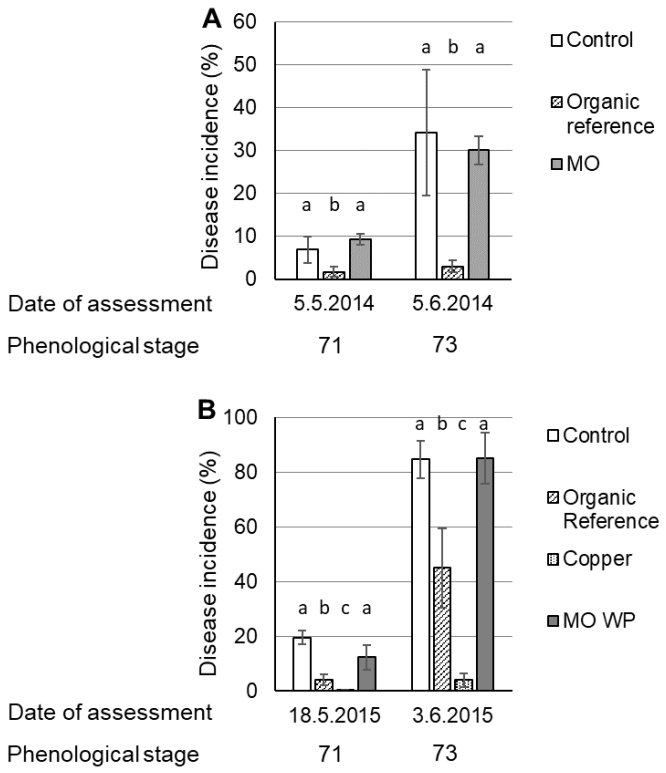


Fig. 5.

Table1

Table 1. Minimal inhibitory concentrations (MIC_{100}) of *Magnolia officinalis* bark extract (MO), magnolol, and honokiol against *Plasmopara viticola, Venturia inaequalis,* and *Phytophthora infestans* in *in vitro* experiments

Pathogen	MIC_{100} MO (μg mL^{-1})			MIC_{100} pure compounds (μg mL^{-1})	
	whole extract	equivalents in extract		magnolol	honokiol
		magnolol†	honokiol‡		
P. viticola	12 (8;17)§ n=7$	1.6	0.5	3 (2;4) n=4	1 (0.9;1.5) n=4
V. inaequalis	32 (32;32) n=6	4.2	1.4	4 (2;6) n=3	3 (2;4) n=3
P. infestans	74 (53;104) n=4	9.8	3.2	11 (8;17) n=4	5 (3;8) n=3

† Equivalents magnolol = MIC_{100} of whole MO extract × 13.3 (content of magnolol in MO extract) × 100^{-1}; ‡ Equivalents honokiol = MIC_{100} of whole MO extract × 4.3 (content of honokiol in MO extract) × 100^{-1}; § Mean with upper and lower limits of the 95% confidence interval; $ Number of independent experiments

Table 2. Efficacy of a *Magnolia officinalis* bark extract (MO) (1 mg mL^{-1}) on grapevine seedlings (*cv.* 'Chasselas') against *Plasmopara viticola* under controlled conditions, compared to efficacies of two concentrations of a copper reference treatment (Cu^{2+} in the form of copper hydroxide). The table shows results (mean ± SD) of five independent experiments, each experiment with six replicate plants per treatment.

	Efficacy (%)[†]			Severity control (%)[‡]
	MO	Cu^{2+}		
	1 mg mL^{-1}	0.3 mg mL^{-1}	0.03 mg mL^{-1}	
Exp_1	89 ± 13	100 ± 0.0	76 ± 17	65 ± 16
Exp_2	99 ± 2	99.6 ± 0.7	99.4 ± 0.5	77 ± 22
Exp_3	96 ± 8	99.8 ± 0.5	86 ± 13	39 ± 9
Exp_4	99.7 ± 0.7	98 ± 4	97 ± 3	72 ± 20
Exp_5	99 ± 2	90 ± 8	83 ± 12	94 ± 7
Mean[§]	**97 ± 5**	**97 ± 4**	**88 ± 10**	**69 ± 20**

[†] Percentage reduction in the diseased leaf area in treated plants compared to the non-treated control; [‡] Percentage leaf area with disease symptoms in non-treated control plants; [§] Mean ± SD of independent experiments

Table 3. Efficacy of a *Magnolia officinalis* bark extract (MO) (1 mg mL^{-1}) on apple seedlings (*cv.* 'Rubinette') against *Venturia inaequalis* under controlled conditions, compared to efficacies of two concentrations of a copper reference treatment (Cu^{2+} in the form of copper hydroxide). The table shows results (mean ± SD) of four independent experiments, each experiment with six replicate plants per treatment.

	Efficacy (%)†			Severity control (%)‡
	MO	Cu^{2+}		
	1 mg mL^{-1}	0.3 mg mL^{-1}	0.03 mg mL^{-1}	
Exp_1	96 ± 4	86 ± 12	62 ± 18	23 ± 14
Exp_2	97 ± 3	92 ± 4	88 ± 9	36 ± 5
Exp_3§	85 ± 9	97 ± 2	87 ± 7	13 ± 4
Exp_4§	93 ± 3	87 ± 9	84 ± 15	28 ± 14
Mean$	**93 ± 5**	**91 ± 5**	**80 ± 12**	**25 ± 10**

† Percentage reduction in the diseased leaf area in treated plants compared to the non-treated control; ‡ Percentage leaf area with disease symptoms in non-treated control plants; § MO in a formulation; $ Mean ± SD of independent experiments

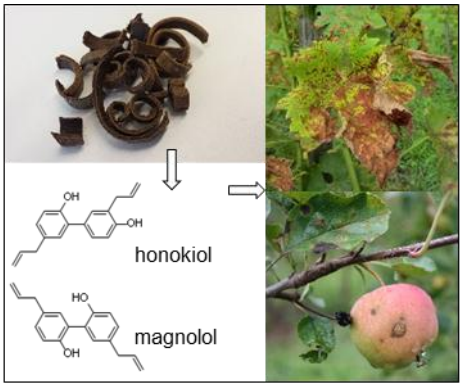
Table4

Table 4. Efficacy of a *Magnolia officinalis* bark extract (MO) on tomato seedlings (*cv.* 'Marmande') against *Phytophthora infestans* under controlled conditions, compared to efficacies of two concentrations of a copper reference treatment (Cu^{2+} in the form of copper hydroxide). The table shows results (mean ± SD) of one to three independent experiments, each experiment with six replicate plants per treatment.

	Efficacy (%)†						Severity control (%)‡
	MO (mg mL^{-1})				Cu^{2+}		
	4	2	1	0.5	0.3	0.03	
Exp_1	82 ±11	42 ± 22	46 ± 17		75 ± 23	59 ± 28	85 ± 9
Exp_2		64 ± 11	33 ± 12	32 ± 8	77 ± 17	72 ± 23	93 ± 13
Exp_3		91 ± 5	77 ± 18	60 ± 12	97 ± 1	94 ± 2	53 ± 5
Mean§	**82**	**66 ± 25**	**52 ± 22**	**46 ± 20**	**83 ± 12**	**75 ± 18**	**77 ± 21**

† Percentage reduction in the diseased leaf area in treated plants compared to the non-treated control; ‡ Percentage leaf area with disease symptoms in non-treated control plants; § Mean ± SD of independent experiments

Graphical Abstract



3.4. Eudesmane Sesquiterpenes from *Verbesina lanata* with Inhibitory Activity against Grapevine Downy Mildew

Justine Ramseyer, Barbara Thuerig, Maria De Mieri, Hans-Jakob Schärer, Thomas Oberhänsli, Mahabir P. Gupta, Lucius Tamm, Matthias Hamburger, and Olivier Potterat

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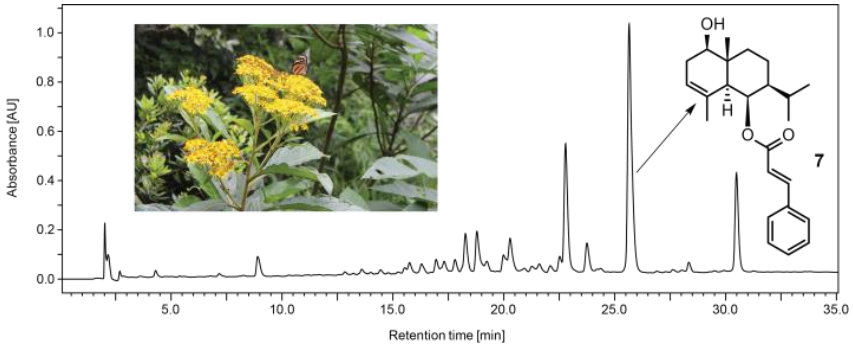

The ethyl acetate extract from inflorescences of *Verbesina lanata* (Asteraceae) showed significant inhibitory activity *in vitro* against *Plasmopara viticola*. *In vivo* activity was assessed on grapevine seedlings and leaf surface infestation was lowered by 82% at a concentration of 1 mg/mL. With the aid of HPLC-based activity profiling, the activity could be correlated with a series of lipophilic compounds. Preparative isolation by a combination of chromatographic techniques, including silica gel column chromatography and preparative HPLC, afforded 16 eudesmane sesquiterpenes including eight new congeners. Nine compounds were obtained in sufficient quantities to be tested *in vitro*, and were found to potently inhibit the growth of *P. viticola*.

Extraction of plant material, HPLC-microfractionation, preparative fractionation and isolation of active compounds, recording and interpretation of the data (except HR-MS) for structural elucidation together with Maria De Mieri, writing the manuscript draft, and preparing the figures were my contributions to this publication.

Justine Fabienne Ramseyer

JOURNAL OF NATURAL PRODUCTS

Article

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Eudesmane Sesquiterpenes from *Verbesina lanata* with Inhibitory Activity against Grapevine Downy Mildew

Justine Ramseyer,[†] Barbara Thuerig,[‡] Maria De Mieri,[†] Hans-Jakob Schärer,[‡] Thomas Oberhänsli,[‡] Mahabir P. Gupta,[§] Lucius Tamm,[‡] Matthias Hamburger,[†] and Olivier Potterat*[,†]

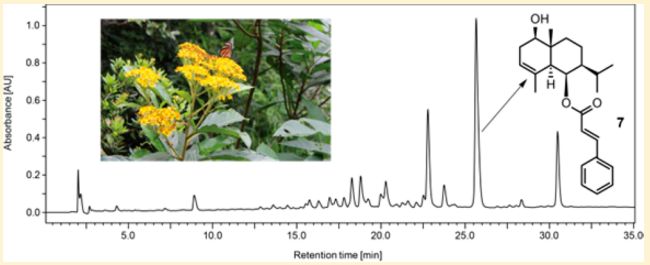
[†]Division of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel, Klingelbergstrasse 50, 4056 Basel, Switzerland

[‡]Research Institute of Organic Agriculture FiBL, Department of Crop Sciences, Ackerstrasse 113, 5070 Frick, Switzerland

[§]Center for Pharmacognostic Research on Panamanian Flora, Faculty of Pharmacy, University of Panama, Panama City, Republic of Panama

Supporting Information

ABSTRACT: An in-house library of more than 3000 extracts of plant and fungal origin was screened against some major plant pathogens. As one of the hits, an ethyl acetate extract from inflorescences of *Verbesina lanata* showed significant inhibitory activity in vitro against grapevine downy mildew (*Plasmopara viticola*), with a MIC_{100} value of 35 μg/mL. An emulsifiable concentrate formulation with 50 mg/g of the extract was developed for in vivo evaluation. A suspension of the formulation containing 1 mg/mL of extract lowered leaf surface infection of grapevine seedling by 82% compared to the nontreated control. With the aid of HPLC-based activity profiling, the antifungal activity was correlated with a series of lipophilic compounds. Preparative isolation by a combination of chromatographic techniques afforded 16 eudesmane sesquiterpenes including eight new congeners. Nine compounds were obtained in sufficient quantities to be tested in vitro and were found to inhibit the zoospore activity of *P. viticola* with MIC_{100} values ranging from 4 to 50 μg/mL. The two major compounds, 6β-cinnamoyloxy-4β,9β,15-trihydroxyeudesmane (**9**) and 6β-cinnamoyloxy-1β,15-dihydroxyeudesm-4-en-3-one (**13**), showed MIC_{100} values of 5 and 31 μg/mL, respectively.



Plasmopara viticola (Berk. & M. A. Curtis) Berl. & de Toni, known as grapevine downy mildew, is an obligately biotrophic oomycete that infects all green parts of grapevine plants. The sporangia are spread by windblown rain. Under weather conditions favorable for the pathogen, up to 100% of the yield can be lost if plants are left untreated.[1]

Copper salts are widely used to fight against a wide range of plant pathogens.[2] Their use in agriculture was established in the 1880s when the French scientist Millardet demonstrated that spraying vineyards with a mixture of copper sulfate, lime, and water (Bordeaux mixture) drastically reduced infection of grapevine by downy mildew.[3] The use of copper is still permitted in conventional and organic production systems,[4] but its utilization is increasingly criticized due to an unfavorable ecotoxicological profile.[2,5,6] Copper accumulates in soils and is potentially toxic to some nontarget organisms.[7−10]

Natural products, especially plant extracts, could serve as sustainable and environmentally friendly alternatives since they are typically rapidly degraded under field conditions.[11−15] Promising plant extracts with reported activity against *P. viticola* include *Yucca schidigera*, *Salvia officinalis*, *Inula viscosa*, *Glycyrrhiza glabra*, *Larix decidua*, *Juncus effusus*, and *Vitis vinifera*.[4,14,16−20]

In an ongoing search for safer replacements of copper fungicides, an in-house library comprising over 3000 extracts of plant and fungal origin (10 mg/mL in DMSO) was screened for in vitro inhibitory activity against grapevine downy mildew.[20,21] As one of the hits, an ethyl acetate extract from inflorescences of *Verbesina lanata* B. L. Rob. & Greenm. (Asteraceae) showed pronounced activity (MIC_{100} of 35 μg/mL). The genus *Verbesina*, commonly known as "crownbeard", comprises over 300 species of herbs, shrubs, and trees bearing numerous bright yellow flowerheads.[22−24] *V. lanata* is distributed in Central America[22] and has not been phytochemically investigated up to now.

We here report on the isolation and structure elucidation of 16 eudesmane sesquiterpenes (**1**−**16**), including eight new congeners, with strong inhibitory activity against *P. viticola*.

RESULTS AND DISCUSSION

Compound Isolation and Structure Elucidation. The ethyl acetate extract of *V. lanata* flowerheads was fractionated by silica gel column chromatography. Out of a total of 26

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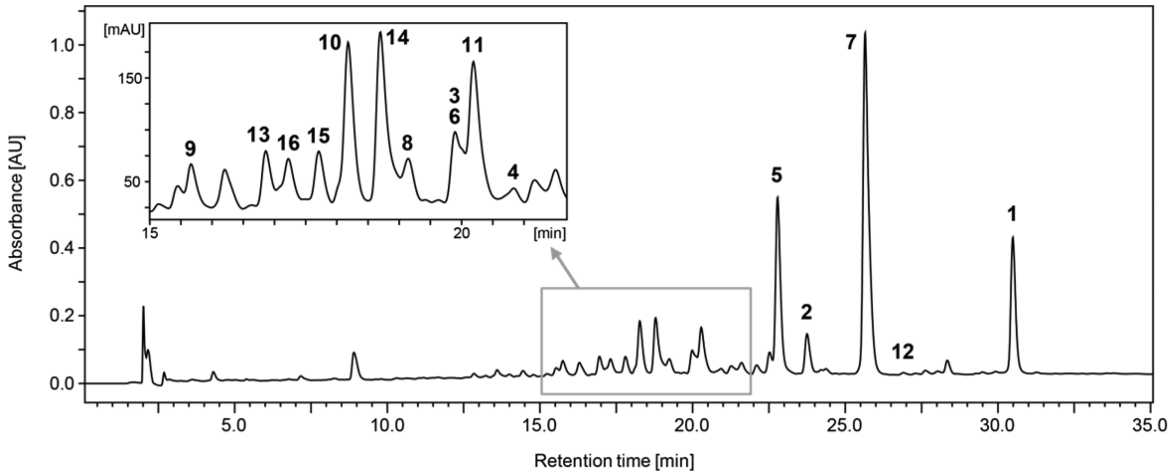

Figure 1. HPLC-PDA analysis of the ethyl acetate extract of *V. lanata*. SunFire C_{18} column; A: water + 0.1% formic acid, B: acetonitrile + 0.1% formic acid; 30% to 100% B in 30 min, and 100% B for 5 min; detection at 254 nm. Bolded numerals refer to isolated compounds.

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds 9−12 ($CDCl_3$; 500 MHz for ^{1}H, 125 MHz for ^{13}C; δ in ppm)

	9		10		11		12[a]	
position	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type
1	2.01, dt (12.8, 2.8), β	39.5, CH_2	3.85, dd (11.9, 4.3), α	75.1, CH	3.11, d (9.2), α	84.6, CH	3.41, dd (11.6, 4.0), α	78.2, CH
	1.16, m,[b] α							
2	1.69, m[b]	20.8, CH_2	1.98, ddd (13.7, 4.3, 2.8), α	37.1, CH_2	3.61, ddd (11.0, 9.2, 5.7), β	70.5, CH	1.66, m[b]	26.7, CH_2
	1.69, m[b]		1.73, m,[b] β				1.66, m[b]	
3	1.70, m[b], β	35.8, CH_2	4.33, br m, β	74.4, CH	2.62, dd (13.2, 5.7), β	43.1, CH_2	2.17, m, α	32.5, CH_2
	1.54, td (14.0, 4.2), α				2.11, m[b], α		2.09, m,[b] β	
4		73.6, C		147.4, C		142.8, C		132.4, C
5	1.31, br s, α	49.0, CH	2.48, br s, α	46.5, CH	1.99, br s, α	51.9, CH		131.7, C
6	5.71, br s, α	70.8, CH	5.74, br s, α	71.3, CH	5.79, br s, α	70.9, CH	6.13, br d (1.8), α	71.2, CH
7	1.10, m[b], α	49.8, CH	1.17, m, α	50.4, CH	1.13, m, α	50.2, CH	1.06, m, α	48.8, CH
8	1.89, qd (11.6, 3.5), β	26.4, CH_2	1.72, m,[b] α	20.6, CH_2	1.68, dq (13.0, 2.8), α	20.2, CH_2	1.73, m[b]	20.4, CH_2
	1.61, m,[b] α		1.60, m, β		1.58, qd (13.0, 3.4), β		1.73, m[b]	
9	3.21, dd (11.6, 3.5), α	80.3, CH	2.04, dt (13.1, 3.5), β	37.3, CH_2	2.10, m[b], β	37.5, CH_2	2.07, m,[b] β	38.5, CH_2
			1.30, td (13.1, 3.5), α		1.24, td (13.0, 3.4), α		1.19, m,[b] α	
10		39.4, C		40.7, C		39.5, C		38.3, C
11	1.45, m	28.8, CH	1.36, m	28.2, CH	1.38, m	28.1, CH	1.58, m	29.0, CH
12	0.87, d (6.7)	20.6, CH_3	1.04, br d (6.5)	22.1, CH_3	0.87, d (6.7)	20.5, CH_3	0.96, d (7.3)	20.7, CH_3
13	0.93, d (6.7)	21.6, CH_3	0.87, br d (6.5)	20.5, CH_3	1.04, d (6.7)	22.1, CH_3	0.94, d (7.3)	20.7, CH_3
14	1.38, s	14.3, CH_3	1.00, s	12.5, CH_3	1.04, br s	14.4, CH_3	1.14, br s	18.3, CH_3
15	3.78, d (10.4)	69.0, CH_2	5.03, br s	112.8, CH_2	4.87, br s	110.9, CH_2	1.88, s	19.3, CH_3
	3.60, d (10.4)		4.88, br s		4.78, br s			
1′		167.5, C		167.0, C		166.9, C		165.7, C
2′	6.41, d (16.0)	118.5, CH	6.41, d (16.0)	118.5, CH	6.39, d (15.9)	118.4, CH	6.40, d (16.2)	118.8, CH
3′	7.67, d (16.0)	145.5, CH	7.70, d (16.0)	145.2, CH	7.68, d (15.9)	145.2, CH	7.65, d (16.2)	144.1, CH
4′		134.4, C		134.4, C		134.4, C		134.3, C
5′, 9′	7.51, m	128.4, CH	7.53, m	128.3, CH	7.51, m	128.2, CH	7.51, m	127.8, CH
6′, 8′	7.36, m[b]	129.0, CH	7.39, m[b]	129.0, CH	7.36, m[b]	129.0, CH	7.36, m[b]	128.6, CH
7′	7.36, m[b]	130.6, CH	7.39, m[b]	130.5, CH	7.36, m[b]	130.5, CH	7.36, m[b]	129.8, CH

[a] ^{13}C NMR data extracted from $^{1}H-^{13}C$ 2D inverse-detected experiments. [b] Overlapping signals.

fractions, five fractions showed strong antifungal activity against *P. viticola* in vitro (data not shown). HPLC microfractionation combined with bioactivity assessment in a process referred to as HPLC-based activity profiling[21] enabled the activity to be correlated with a group of peaks (Figure S1, Supporting Information) showing strong UV absorption maxima at 280 nm. Targeted isolation by a combination of preparative and semipreparative HPLC afforded compounds **1**−**16**, which were shown to account for most of the peaks in the HPLC-UV chromatographic trace (Figure 1).

Compounds **1**−**8** were identified by NMR spectroscopic data analysis and comparison with literature values. Compounds **1**−

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8 were found to be known eudesmane sesquiterpenes with cinnamoyloxy groups attached at C-6. They were identified as 6*β*-cinnamoyloxy-4*β*-hydroxyeudesmane (**1**),[25] 6*β*-cinnamoyloxy-3*β*,4*α*-dihydroxyeudesmane (**2**),[26] 6*β*-cinnamoyloxy-3*α*-hydroperoxy-1*β*-hydroxyeudesm-4(15)-ene (**3**),[27] 6*β*-cinnamoyloxy-3*α*-hydroperoxy-1*β*-hydroxyeudesm-4-ene (**4**),[27] 6*β*-cinnamoyloxy-1*β*-hydroxyeudesm-4-en-3-one (**5**),[27] 6*β*-cinnamoyloxy-1*β*,3*β*-dihydroxyeudesm-4-ene (**6**),[26] 6*β*-cinnamoyloxy-1*β*-hydroxyeudesm-3-ene (**7**),[27] and 7-epi-6*α*-cinnamoyloxy-1*β*,4*α*-dihydroxyeudesmane (**8**).[26] These compounds have been previously isolated from species of the genus *Verbesina* and/or from *Brintonia discoidea*, but they are reported here for the first time from *V. lanata*. Some minor inconsistencies were detected in the previously reported NMR data, and some assignments were therefore revised. Full ^{1}H and ^{13}C NMR spectroscopic assignments of **1**−**8** are provided as Supporting Information.

Compound **9** was obtained as a pale yellow oil. Its molecular formula was established as $C_{24}H_{34}O_5$ from the $[M + Na]^+$ sodium adduct ion at *m*/*z* 425.2297 (calcd for $C_{24}H_{34}NaO_5^+$, 425.2299) in the HRESIMS and corroborated by the ^{13}C NMR data. The ^{13}C NMR spectrum showed the presence of signals for three methyls, five methylenes (including one oxygenated carbon (δ_C 69.0)), 12 methines (including seven olefinic and aromatic carbons), one oxygenated tertiary carbon (δ_C 73.6), one carbonyl (δ_C 167.5), and two quaternary carbons (δ_C 39.4 and 134.4). The ^{1}H and ^{13}C NMR data were fully assigned by 2D NMR ($^{1}H-^{1}H$, COSY, HSQC, and HMBC) experiments (Table 1). Comparison of the NMR data with those of compounds **1** and **2** indicated that **9** also possesses a *trans*-decalin eudesmane skeleton with an axial (*β*) cinnamoyloxy substituent at C-6. A hydroxy group was located at C-9 (δ_H 3.21, δ_C 80.3), as shown by a COSY correlation between H_2-8 (δ_H 1.89 and 1.61) and H-9. HMBC correlations of H-5 (δ_H 1.31) and H_2-15 (δ_H 3.78 and 3.60) to δ_C 73.6 allowed the assignment of the oxygenated tertiary carbon as C-4 (δ_C 73.6). By HMBC correlations of H_2-15 (δ_H 3.78 and 3.60) to C-3 (δ_C 35.8), C-4 (δ_C 73.6), and C-5 (δ_C 49.0), the position of the oxygenated methylene (δ_C 69.0) was assigned at C-15. The relative configuration was established from $^{1}H-^{1}H$ coupling constants and NOESY correlations (Figure 2). Thus, the

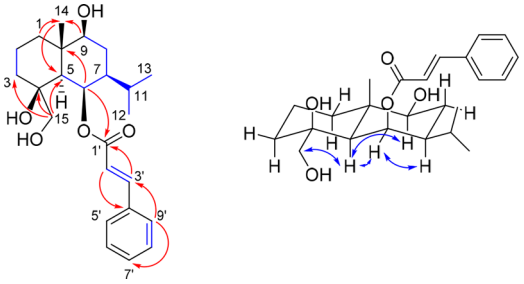
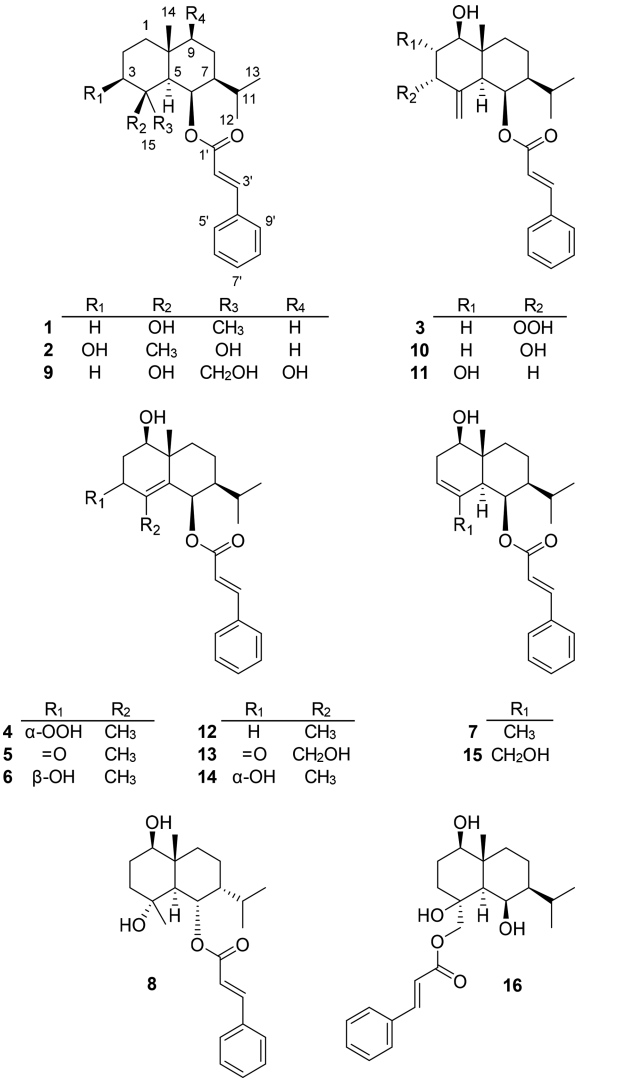


Figure 2. Key COSY (blue bonds), HMBC (red arrows), and NOESY correlations (blue arrows) of compound **9**.

hydroxy group at C-9 was determined as equatorial due to the diaxial coupling of H-9 (δ_H 3.21, dd, *J* = 11.6/3.5 Hz) with H-8$_{ax}$ (δ_H 1.89, qd, *J* = 11.6/3.5 Hz). A NOESY correlation of H-9 and H-5 (δ_H 1.31) confirmed the alpha-orientation of H-9. Similarly, the orientation of C-15 (δ_H 3.78 and 3.60) was assigned as alpha by the NOESY correlation of H_2-15 and H-5 (δ_H 1.31). Thus, the structure of **9** was established as 6*β*-cinnamoyloxy-4*β*,9*β*,15-trihydroxyeudesmane.



Compound **10** was obtained as a pale yellow, amorphous solid. It had a molecular formula of $C_{24}H_{32}O_4$ as determined from a $[M + Na]^+$ ion at *m*/*z* 407.2192 in the HRESIMS (calcd for $C_{24}H_{32}NaO_4^+$, 407.2193) and thus differed by 16 units from that of **3**. The NMR data of **10** (Table 1) closely resembled those of **3**. The only remarkable difference was an upfield shift of C-3 in **10** (δ_H 4.33, δ_C 74.4; vs δ_H 4.44, δ_C 87.0 in **3**), which suggested the replacement of the peroxide at C-3 by a hydroxy group. Key NMR correlations for **10** are available as Supporting Information (Figure S24). Accordingly, the compound was assigned as 6*β*-cinnamoyloxy-1*β*,3*α*-dihydroxyeudesm-4(15)-ene.

Compound **11**, isolated as a yellow oil, had the same molecular formula ($C_{24}H_{32}O_4$) as **10**, as determined by the HRESIMS $[M + H]^+$ ion at *m*/*z* 385.2373 (calcd for $C_{24}H_{33}O_4^+$, 385.2373). The NMR data indicated that **11** is a positional isomer of the latter. Comparison of ^{1}H, ^{13}C, and 2D NMR data (Table 1) suggested that the hydroxy group in **11** had to be located C-2. This assignment was corroborated by key COSY correlations of H-2 (δ_H 3.61) with H-1 (δ_H 3.11) and H_2-3 (δ_H 2.62 and 2.11). The *α*-equatorial orientation of OH-2 was deduced from the diaxial coupling of H-2 with H-1 and H-3 ($J_{H_1-H_2}$ = 9.2 Hz, and $J_{H_2-H_3}$ = 11.0 Hz, respectively) and confirmed by the NOESY correlation of H_3-14 with H-2ax (Figure S32, Supporting Information). Therefore, the structure

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Table 2. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds 13−16 ($CDCl_3$; 500 MHz for ^{1}H, 125 MHz for ^{13}C; δ in ppm)

	13[a]		14		15		16	
position	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type
1	3.79, dd (10.1, 7.6), α	74.5, CH	3.70, dd (13.0, 3.5), α	73.5, CH	3.54, dd (10.0, 6.1), α	76.4, CH	3.22, dd (12.0, 3.5), α	79.7, CH
2	2.62, m[b]	42.5, CH_2	1.89, td (13.0, 4.0), β	36.0, CH_2	2.33, m[b]	31.9, CH_2	2.01, qd (12.0, 3.5), β	26.4, CH_2
	2.62, m[b]		1.82, ddd (13.0, 3.5, 1.7), α		2.00, m[b]		1.67, m, α	
3		198.5, C	4.06, br d (4.0), β	71.0, CH	5.63, br m	124.4, CH	1.78, dd (12.0, 3.5), β	34.2, CH_2
							1.63, m,[b] α	
4		136.2, C		132.9, C		136.6, C		73.8, C
5		157.5, C		136.1, C	2.33, m,[b] α	48.3, CH	1.18 br m, α	48.3, CH
6	6.33, d (2.1), α	70.2, CH	6.10, d (2.1), α	71.1, CH	5.91, br s, α	70.6, CH	4.44, br s, α	69.2, CH
7	1.26, m, α	48.4, CH	1.11, m,[b] α	48.7, CH	1.21, m,[b] α	49.1, CH	0.88, m, α	49.5, CH
8	1.85, m[b]	19.9, CH_2	1.74, m[b]	20.5, CH_2	1.70, m[b], α	20.4, CH_2	1.63, m[b], α	20.4, CH_2
	1.85, m[b]		1.74, m[b]		1.63, qd (13.1, 3.5), β		1.52, qd (13.0, 3.0), β	
9	2.24, dt (13.1, 3.4), β	37.5, CH_2	2.10, dt (13.1, 3.0), β	38.4, CH_2	2.01, dt (13.1, 3.5), β	35.5, CH_2	1.94, dt (13.0, 3.0), β	39.3, CH_2
	1.38, td (13.1, 5.8), α		1.23, m, α		1.19, m[b], α		1.06, br t (13.0), α	
10		41.2, C		39.6, C		37.7, C		38.6, C
11	1.68, m	29.0, CH	1.59, m	29.1, CH	1.43, m	28.7, CH	1.61, m[b]	28.7, CH
12	1.00, d (5.2)	20.6, CH_3	0.95, d (6.7)	21.0, CH_3	0.86, d (6.7)	20.3, CH_3	0.97, d (7.5)	21.1, CH_3
13	0.98, d (5.2)	20.6, CH_3	0.94, d (6.7)	21.0, CH_3	1.01, d (6.7)	22.1, CH_3	0.96, d (7.5)	20.9, CH_3
14	1.32, s	16.4, CH_3	1.10, s	17.1, CH_3	1.07, br s	12.3, CH_3	1.37, br s	14.8, CH_3
15	4.63, d (12.5)	55.3, CH_2	2.04, s	17.4, CH_3	4.21, br d (12.0)	65.0, CH_2	4.30, d (11.3)	70.2, CH_2
	4.56, d (12.5)				3.95, br d (12.0)		4.13, d (11.3)	
1′		166.2, C		166.2, C		166.9, C		167.0, C
2′	6.40, d (16.0)	117.1, CH	6.40, d (15.9)	118.6, CH	6.39, d (15.9)	118.4, CH	6.45, d (16.2)	117.6, CH
3′	7.70, d (16.0)	146.1, CH	7.65, d (15.9)	144.8, CH	7.68, d (15.9)	145.2, CH	7.70, d (16.2)	145.7, CH
4′		134.0, C		134.5, C		134.4, C		134.3, C
5′, 9′	7.53, m	128.1, CH	7.51, m	128.2, CH	7.50, m	128.3, CH	7.53, m	128.3, CH
6′, 8′	7.39, m[b]	128.7, CH	7.36, m[b]	129.0, CH	7.36, m[b]	129.0, CH	7.38, m[b]	129.1, CH
7′	7.39, m[b]	130.5, CH	7.36, m[b]	130.4, CH	7.36, m[b]	130.5, CH	7.38, m[b]	130.7, CH

[a] ^{13}C extracted from $^{1}H-^{13}C$ 2D inverse-detected experiments. [b] Overlapping signals.

of **11** was established as 6β-cinnamoyloxy-1β,2α-dihydroxyeudesm-4(15)-ene.

Compound **12** was isolated as a colorless oil. The HRESIMS $[M + H]^+$ ion at m/z 369.2427 (calcd for $C_{24}H_{33}O_3^+$, 369.2424) established a molecular formula of $C_{24}H_{32}O_3$. Comprehensive analysis of its NMR data (Table 1) showed that **12** possesses a similar structure to those of **4**−**6**, with a double bond between C-4 and C-5. The only difference was at C-3, which appeared as a methylene (δ_H 2.17 and 2.09, δ_C 32.5), instead of an oxygen-bearing carbon. This was confirmed by a COSY correlation of H_2-2 (δ_H 1.66, overl.) with H_2-3 and by an HMBC correlation of H_3-15 (δ_H 1.88) with C-3 (δ_C 32.5) (Figure S39, Supporting Information). Thus, the structure of compound **12** was elucidated as 6β-cinnamoyloxy-1β-hydroxyeudesm-4-ene.

Compound **13** was obtained as a white solid. It showed a $[M + Na]^+$ adduct ion at m/z 421.1984 (calcd for $C_{24}H_{30}NaO_5^+$, 421.1986), corresponding to a molecular formula of $C_{24}H_{30}O_5$. By comparison of ^{1}H, ^{13}C, and 2D NMR data (Table 2) with those of the other isolated compounds, **13** proved to be structurally similar to **5**, with the only difference being that the methyl group at C-15 (**5**: δ_H 2.03, δ_C 11.0) was replaced by an oxygenated methylene (**13**: δ_H 4.63 and 4.56, δ_C 55.3). This assignment was corroborated by HMBC correlations of H_2-15 with C-3 (δ_C 198.5) and C-5 (δ_C 157.5) (Figure S46, Supporting Information). Hence, **13** was assigned as 6β-cinnamoyloxy-1β,15-dihydroxyeudesm-4-en-3-one.

Compound **14** was isolated as a yellow oil. Its molecular formula of $C_{24}H_{32}O_4$ was deduced from the HRESIMS $[M + H]^+$ ion at m/z 385.2361 (calcd for $C_{24}H_{33}O_4^+$, 385.2373) and from the ^{13}C NMR data. Comprehensive analysis of its NMR data (Table 2) indicated the same planar structure as for compound **6**. The relative configuration of both compounds was found to differ only in the orientation of the hydroxy group at C-3. For compound **6**, a J coupling constant for H-3/H-2 of 7.0 Hz corresponded to a dihedral angle of about 150° and suggested a β-orientation of the hydroxy group attached at C-3. This was also supported by the NOESY correlation of H-3 (δ_H 4.10, apparent q, J = 7.0 Hz) with H-2_{eq} (δ_H 2.11, ddd, J = 11.5/7.0/3.0 Hz) (Figure S7, Supporting Information). In compound **14**, the J coupling constant for H-3/H-2 of 4.0 Hz, arising from a dihedral angle of ca. 50°, indicated an alpha orientation of OH-3. This assignment was in agreement with the NOESY correlation of H-2_{ax} (δ_H 1.89) and H-3 (δ_H 4.06, br d, J = 4.0 Hz) (Figure S54, Supporting Information). Thus, the structure of **14** was established as 6β-cinnamoyloxy-1β,3α-dihydroxyeudesm-4-ene. Jakupovic et al.[28] previously reported H-3 (δ_H 4.08) in cinnamoyloxy-1β,3β-dihydroxyeudesm-4-ene (**6**) as a broad doublet with a coupling constant of 4 Hz. According to the NMR data of both epimers reported here, it seems likely that Jakupovic et al.[26] isolated in fact 6β-cinnamoyloxy-1β,3α-dihydroxyeudesm-4-ene and reported it erroneously as 6β-cinnamoyloxy-1β,3β-dihydroxyeudesm-4-ene.

Compound **15** was obtained as a yellow oil. Its HRESIMS showed an $[M + Na]^+$ adduct ion at m/z 407.2192 (calcd for $C_{24}H_{32}NaO_4^+$, 407.2193), corresponding to a molecular formula of $C_{24}H_{32}O_4$. Analysis of the NMR data (Table 2)

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revealed a strong resemblance to **7**. The only difference was at C-15, which was an oxygenated methylene (**15**: δ_H 4.21 and 3.95, δ_C 65.0) instead of a methyl group (**7**: δ_H 1.70, δ_C 20.6). This was confirmed by HMBC correlations of H_{2a}-15 (δ_H 3.95) with C-3 (δ_C 124.4) and C-5 (δ_C 48.3) (Figure S62, Supporting Information). Thus, the structure of compound **15** was determined as 6*β*-cinnamoyloxy-1*β*,15-dihydroxyeudesm-3-ene.

Compound **16**, a yellow solid, gave a molecular formula of $C_{24}H_{34}O_5$ as established by the $[M + Na]^+$ ion at *m*/*z* 425.2295 (calcd for $C_{24}H_{34}NaO_5^+$, 425.2299) in the HRESIMS. It was thus an isomer of compound **9**. The ^{13}C NMR spectrum showed the same multiplicities as for **9**, suggesting the presence of a *trans*-decalin eudesmane skeleton with a cinnamoyloxy group. The ^1H and ^{13}C NMR data (Table 2) were fully assigned by 2D NMR ($^1H-^1H$ COSY, HSQC, and HMBC) experiments and revealed some differences between **9** and **16**. First, key HMBC correlations of H_2-15 (δ_H 4.30 and 4.13) to the carbonyl C-1′ (δ_C 167.0) inferred the attachment of the cinnamoyloxy group at H_2-15 (Figure 3). Consequently, H-6

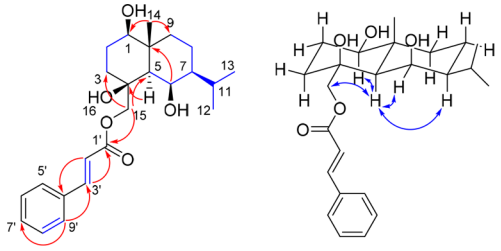

Figure 3. Key COSY (blue bonds), HMBC (red arrows), and NOESY correlations (blue arrows) of compound **16**.

was shifted upfield (δ_H 4.44, br s, δ_C 69.2) compared to the corresponding resonances in the cinnamoyl eudesmane derivatives **1**−**15**. Further differences from compound **9** were the presence of a hydroxy group at C-1 (δ_H 3.22, δ_C 79.7) and of a methylene at C-9 (δ_H 1.94 and 1.06, δ_C 39.3). The relative configuration of **16** was established as follows. Characteristic ^{13}C NMR shifts of C-14, C-10, and C-5[28] and 1,3-diaxial NOESY correlations indicated a *trans* junction of the decalin ring system (Figure 3). The *β*-equatorial orientation of the hydroxy group at C-1 was supported by the diaxial coupling H-1/H-2_{axial} (J = 12.0 Hz). The multiplicities of H-6 (δ_H 4.44, br s) and H-7 (δ_H 0.88, m) were similar to those found in compounds **1**−**15** and indicated their beta cofacial orientation. Finally, the configuration at C-4 was deduced from the NOESY contacts of H_2-15 and H-5. Compound **16** was thus assigned as 15-cinnamoyloxy-1*β*,4*β*,6*β*-trihydroxyeudesmane.

The absolute configuration of compound **12** was assigned by electronic circular dichroism (ECD). The experimental ECD spectrum of **12** showed a negative Cotton effect at 280 nm due to the $\pi \rightarrow \pi^*$ transition of the cinnamoyl group (Figure 4) and corresponding to a strong UV absorption maximum (Figure S40, Supporting Information). The experimental ECD spectrum of **12** matched well with the ECD curve calculated for the (1*R*,6*R*,7*S*,10*R*) enantiomer (Figure 4). The ECD spectra of compounds **4**−**6**, **13**, and **14** were similar to that of **12**. They all had the same allylic cinnamate group, and the ECD data suggested the same absolute configuration as for **12** (Figures S5, S6, S8, S47, and S55, Supporting Information). The absolute configuration of the remaining compounds was assigned tentatively based on biogenetic considerations and on the assumption that they possess the same absolute

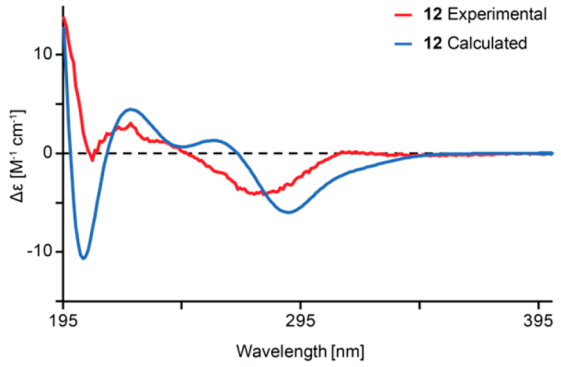

Figure 4. Experimental and calculated ECD spectra of compound **12** (1*R*,6*R*,7*S*,10*R*).

configuration at C-10. This was also in agreement with the reported absolute configuration of **2**, which had been established by X-ray single-crystal analysis.[29]

Antifungal Activity. The crude plant extract showed a minimal inhibitory concentration (MIC_{100}) against *P. viticola* of 35.4 *μ*g/mL, as determined in two independent experiments (Table 3). Owing to this promising activity in vitro, the extract

Table 3. In Vitro Minimal Inhibitory Concentrations (MIC_{100}) of *Verbesina lanata* Extract and Selected Constituents against *Plasmopara viticola*

	MIC_{100} [*μ*g/mL]			
compound	Exp 1[a]	Exp 2	Exp 3	mean[b]
2[c]	6.3	3.1	3.1	3.9
4	12.5	6.3	12.5	9.9
5	3.1	6.3	6.3	5.0
7	25	12.5	100	31.5
8	25	25	12.5	19.8
9	50	50	50	50.0
10[d]	6.3	6.3	12.5	7.9
14	12.5	6.3	12.5	9.9
16	25	50	50	39.7
extract		50	25	35.4

[a]Independent experiments. [b]Data log_2-transformed to calculate mean and retransformed to the linear scale. [c]**1**, **3**, **6**, **11**−**13**, and **15** were not tested due to the insufficient amounts available. [d]Tested sample had ca. 70% purity.

was then tested on grapevine seedlings. In a first attempt, however, no activity was detected, and the lack of activity was found to be due to insufficient solubility (data not shown). To overcome this issue, an emulsifiable concentrate formulation with 50 mg/g of extract was developed (VL-EC). At concentrations of 1 and 0.125 mg/mL of extract, the efficacies were 82% and 73%, respectively, as expressed as the lowering of infected leaf surface in the treated set of seedlings compared to the nontreated control set (disease severity of 92 ± 5%) (Figure 5).

Compounds **2**−**5**, **7**−**10**, **14**, and **16** were available in sufficient amounts to be tested against *P. viticola* in vitro (Table 3). Compounds **2**, **4**, **5**, **10**, and **14** exhibited MIC_{100} values of <10 *μ*g/mL and thus were significantly more active than the extract. These data confirmed that eudesmane sesquiterpenes are the antifungal constituents of *V. lanata* extract.

The genus *Verbesina* comprises a large number of species, but only few of these have been investigated phytochemically.

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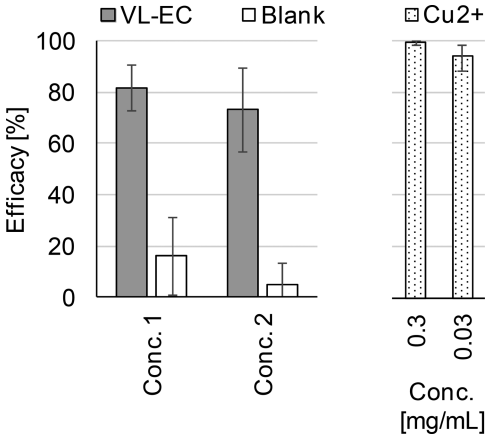

Figure 5. Efficacy of a formulated *Verbesina lanata* extract (VL-EC), blank formulation (Blank), and a copper control (Cu^{2+}) against *Plasmopara viticola* on grapevine seedlings under semicontrolled conditions. VL-EC contained 5% extract and 95% additives. VL-EC and the blank were tested at two concentrations ("Conc. 1": 20 mg/mL VL-EC (1 mg/mL of extract) or 19 mg/mL of formulation additives (blank); "Conc. 2": 2.5 mg/mL VL-EC [0.125 mg/mL of extract or 2.4 mg/mL of formulation additives (blank)]. The disease severity in the control was 92 ± 5%. The figure shows means and standard deviations of one experiment (n = 6).

Isoprenoids, in particular eudesmane sesquiterpenes, are reportedly the characteristic constituents of this genus.[23] Antifungal, antibacterial, and antiviral activity has been described for *Verbesina encelioides*, but the compounds responsible for the activity were not identified.[30] On the other hand, several eudesmane sesquiterpenes that have been isolated from other plants, mostly Asteraceae, have shown antifungal and antibacterial activity against human pathogens.[31−36] However, their efficacy against plant pathogens has not been previously reported. *V. lanata* ethyl acetate extract and five of the isolated compounds showed high inhibitory activity against *P. viticola*. The efficacy was comparable to that of other extracts, such as those from *Juncus effusus* (MIC_{100} 24 μg/mL; active constituent dehydroeffusol, MIC_{100} 4 μg/mL),[20] *Larix decidua* (MIC_{100} 23 μg/mL; active constituents larixyl acetate and larixol, MIC_{100} 6 and 14 μg/mL, respectively),[37] and *Inula viscosa*, *Yucca schidigera*, *Melaleuca alternifolia*, and *Quillaja saponaria* (all >80% efficacy in vivo at 1 mg/mL).[4] In contrast, extracts from *Achillea millefolium*, *Brassica napus*, *Glycyrrhiza glabra*, *Quercus* sp., *Salvia officinalis*, *Solidago virgaurea*, and *Rheum rhabarbarum* showed comparable activity to *V. lanata* at 10- to 15-fold higher concentration only.[4] In conclusion, *V. lanata* extracts and eudesmane sesquiterpenes could provide potential alternatives to copper fungicides, especially in organic farming. However, further studies under field conditions are required to substantiate their potential. The toxicological profiles of the plant extract and pure compounds also need to be investigated to assess the safety of such possible products.

EXPERIMENTAL SECTION

General Experimental Procedures. Formic acid, sulfuric acid, and solvents were obtained from Scharlau (Scharlab S. L.) or from Macron Fine Chemicals (Avantor Performance Materials). HPLC-grade solvents and ultrapure water from a Milli-Q water purification system (Merck Millipore) were used for HPLC. For extraction and preparative separation, technical grade solvents were used after distillation. $CDCl_3$ was purchased from Sigma-Aldrich.

Silica gel 60 F_{254} coated aluminum TLC plates and silica gel (0.063−0.200 mm) for open-column chromatography were obtained from Merck KGaA. TLC plates were visualized under UV light and by spraying with 1% vanillin (Roth GmbH + Co) in EtOH, followed by 10% sulfuric acid in EtOH and heating at 110 °C.

HPLC-PDA-ESIMS analyses were performed on an LC-MS 8030 system (Shimadzu) using a SunFire C_{18} (3.5 μm, 150 × 3.0 mm i.d.) column equipped with a guard column (10 mm × 3.0 mm i.d.) (Waters). LabSolutions software (Shimadzu) was used for data acquisition and processing.

Semipreparative HPLC was performed on an Agilent 1100 Series instrument with a PDA detector. A SunFire C_{18} (5 μm, 150 × 10 mm i.d.) column with a guard column (10 mm × 10 mm i.d.) (Waters) or a Nucleodur CN NP (5 μm, 150 × 10 mm i.d.) column with a guard column (10 mm × 8 mm i.d.) (Macherey-Nagel) was used. Data acquisition and processing was performed using ChemStation software (Agilent Technologies).

Preparative HPLC was carried out on a Puriflash 4100 system (Interchim) or a Reveleris PREP purification system (Büchi). A SunFire C_{18} (5 μm, 150 × 30 mm i.d.) column with guard column (10 mm × 20 mm i.d.) (Waters) was used for separations.

HRESIMS data were recorded in positive ion mode on an Agilent 1290 Infinity system with an Agilent 6540 UHD Accurate-Mass quadrupole time-of-flight detector. Optical rotations were measured in MeOH on a P-2000 digital polarimeter (Jasco) equipped with a sodium lamp (589 nm) and a 10 cm temperature-controlled microcell. UV and ECD spectra were recorded, at a concentration of 0.2 mg/mL in MeOH, on a Chirascan CD spectrometer with 1 mm path precision cells 110 QS (Hellma Analytics). NMR spectra were recorded on a Bruker AVANCE III 500 MHz spectrometer operating at 500 MHz for ^1H and 125 MHz for ^{13}C. The instrument was equipped with a 1 mm TXI microprobe operated at 18 °C or a 5 mm BBO probe at 23 °C (Bruker Biospin). Chemical shifts are reported as δ values (ppm), with residual solvent signal as internal reference, J in Hz. Standard pulse sequences from the Topspin 2.1 software package were used.

Plant Material. Inflorescences of *Verbesina lanata* were collected in November 2001 in Campana, Panama, by CIFLORPAN (Center for Pharmacognostic Research on Panamanian Flora), Panamanian collection number FLORPAN 5456. A voucher specimen is deposited at the Herbarium of the University of Panama. The taxonomic identity was confirmed by Alex Espinosa, botanist at CIFLORPAN. The material was air-dried and minced in Panama. A voucher specimen (no. 948) is also available at the Division of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel.

Extraction and Isolation. Powdered inflorescences (300 g) were mixed with sea sand and percolated sequentially at room temperature with petroleum ether (3.5 L), ethyl acetate (8.6 L), and methanol (6.5 L) to afford, after evaporation under reduced pressure, 8.8 g of petroleum ether extract, 7.6 g of ethyl acetate extract, and 22.8 g of methanol extract. A portion of the ethyl acetate extract (6.8 g) was dissolved in a mixture of dichloromethane and ethyl acetate and adsorbed onto ca. 20 g of silica gel. The dried powder was then loaded onto an open column filled with silica gel (65 cm × 5 cm i.d.). Elution was performed with a step gradient of n-hexane/ethyl acetate [95:5 (2.5 L), 90:10 (2.0 L), 85:15 (1.0 L), 80:20 (3.0 L), 70:30 (2.0 L), 60:40 (2.0 L), 50:50 (2.0 L), 30:70 (2.0 L), and 0:100 (2.5 L)], followed by ethyl acetate/methanol [95:5 (2.0 L), and 80:20 (2.0 L)], at a flow rate of approximately 25 mL/min. A total of 26 fractions (Frs A−Z) were collected based on TLC analysis.

Fraction P (28 mg) was submitted to semipreparative RP-HPLC with a gradient of 40% to 100% acetonitrile in 30 min at a flow rate of 4 mL/min. Repeated injections afforded compound **1** (1.0 mg, t_R 27.8 min). With the aid of preparative RP-HPLC (Puriflash system) compounds **3** (4.0 mg, t_R 18.3 min), **4** (8.5 mg, t_R 19.6 min), **7** (34.9 mg, t_R 26.8 min), and **12** (1.5 mg, t_R 28.3 min) were isolated from a portion (110 mg) of Fr Q (122 mg). Separation was achieved with a gradient of 50% to 100% acetonitrile in 30 min at a flow rate of 20 mL/min. Compound **5** (43.7 mg, t_R 19.2 min) was obtained by preparative RP-HPLC (Reveleris PREP purification system) of a portion (200 mg) of Fr U (500 mg). Separation was achieved with a

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gradient of 50% to 100% acetonitrile in 30 min at a flow rate of 25 mL/min. Compounds **2** (41.7 mg, t_R 28.6 min), **8** (10.1 mg, t_R 23.9 min), and **16** (11.2 mg, t_R 21.6 min) were obtained from a portion (300 mg) of Fr W (304 mg). Separation was achieved by preparative RP-HPLC (Puriflash system), using a gradient of 30% to 100% acetonitrile in 30 min; the flow rate was 20 mL/min. A portion (542 mg) of Fr X (1325 mg) was separated by preparative RP-HPLC (Puriflash system) into 10 subfractions (Frs X1−X10) with 50% acetonitrile for 45 min and a flow rate of 20 mL/min. Subfractions X2, X5, and X7 afforded compounds **9** (6.6 mg, t_R 16.0 min), **15** (8.0 mg, t_R 24.0 min), and **14** (30.6 mg, t_R 29.6 min), respectively. Further purification of subfractions X4, X9, and X10 by semipreparative RP-HPLC afforded compounds **13** (2.2 mg, t_R 37.0 min), **6** (1.1 mg, t_R 48.3 min), and **11** (9.5 mg, t_R 25.5 min), respectively. Separations were performed with 65% B (X4) and 68% (X9) and 73% (X10) methanol at a flow rate of 4 mL/min. About half of Fr X6 (27 mg) was further purified by semipreparative CN NP-HPLC with *n*-heptane/2-propanol (97:3) at a flow rate of 3 mL/min to afford **10** (9.0 mg, t_R 16.0 min). The purity of compounds, as assessed by ^{1}H NMR spectroscopy, was >95% for **2**−**6**, **8**, **10**, **11**, **13**, and **16**; >90% for **1**, **7**, **9**, **12**, and **14**; and >80% for **15**, which contained ca. 20% of another, unidentified, eudesmane derivative.

Microfractionation of Fractions for Activity Profiling. Microfractionation of the active fractions (Frs P, Q, U, W, and X) was carried out by analytical RP-HPLC on an LC-MS 8030 system (Shimadzu) connected with an FC 204 fraction collector (Gilson). For each fraction, a solution of 10 mg/mL was prepared in DMSO. Altogether four injections were performed: 3 × 30 μL with only UV detection (254 nm) for collection (0.9 mg of fraction in total) and 1 × 10 μL with UV-ESIMS detection without collection. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The flow rate was 0.4 mL/min. Gradients applied were as follows: Frs P and Q, 40% to 100% B in 30 min; Fr. U, 40% to 100% B in 40 min; Fr. W, 30% to 100% B in 30 min; Fr. X, 30% to 70% B in 40 min. In each case, the column was washed with 100% B for 5 min. Fractions of 1 min each were collected from minute 2 to the end of the method (Frs P, Q, and W: 33 fractions; U and X: 43 fractions). Microfractions from the three successive injections of a given sample were collected into the corresponding wells of a 96-deep-well plate. Plates were then dried in a Genevac EZ-2 evaporator.

HPLC-PDA-ESIMS Analysis. Analyses were performed on an LC-MS 8030 system (Shimadzu) using a SunFire C_{18} (3.5 μm, 150 × 3.0 mm i.d.) column equipped with a guard column (10 mm × 3.0 mm i.d.) (Waters). The software for data acquisition and processing was LabSolutions (Shimadzu). UV and mass detection ranges were 190 to 600 nm and *m*/*z* 160−1500, respectively. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). A gradient of 30% to 100% B in 30 min was applied, followed by 100% B for 5 min. The flow rate was 0.4 mL/min. The samples were dissolved in DMSO (extract 10 mg/mL, fractions 5 mg/mL), and 10 μL was injected. Compounds were identified in the extract or fractions by comparison of their ESIMS data and retention times with those of the purified compounds.

6β-Cinnamoyloxy-4β-hydroxyeudesmane ***(1):*** colorless oil; $[\alpha]^{25}_D$ −12 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ε) 204 (3.93), 211 (3.86), 217 (3.91), 222 (3.86), 278 (4.00) nm; ^{1}H and ^{13}C NMR, Table S1, Supporting Information; HRESIMS *m*/*z* 393.2399 [M + Na]$^+$ (calcd for $C_{24}H_{34}NaO_3^+$, 393.2400).

6β-Cinnamoyloxy-3β,4α-dihydroxyeudesmane ***(2):*** pale yellow plates (ethyl acetate); $[\alpha]^{25}_D$ −59 (*c* 0.1, MeOH), UV (MeOH) λ_{max} (log ε) 204 (4.19), 217 (4.23), 222 (4.17), 279 (4.41) nm; ^{1}H and ^{13}C NMR, Table S1, Supporting Information; HRESIMS *m*/*z* 409.2347 [M + Na]$^+$ (calcd for $C_{24}H_{34}NaO_4^+$, 409.2349).

6β-Cinnamoyloxy-3α-hydroperoxy-1β-hydroxyeudesm-4(15)-ene ***(3):*** colorless oil; $[\alpha]^{25}_D$ 20 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.19), 217 (4.18), 222 (4.12), 279 (4.32) nm; ^{1}H and ^{13}C NMR, Table S1, Supporting Information; HRESIMS *m*/*z* 423.2130 [M + Na]$^+$ (calcd for $C_{24}H_{32}NaO_5^+$, 423.2142).

6β-Cinnamoyloxy-3α-hydroperoxy-1β-hydroxyeudesm-4-ene ***(4):*** colorless oil; $[\alpha]^{25}_D$ −47 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.35), 217 (4.28), 222 (4.20), 278 (4.36) nm; ^{1}H and ^{13}C NMR, Table S1, Supporting Information; HRESIMS *m*/*z* 423.2134 [M + Na]$^+$ (calcd for $C_{24}H_{32}NaO_5^+$, 423.2142).

6β-Cinnamoyloxy-1β-hydroxyeudesm-4-en-3-one ***(5):*** colorless oil; $[\alpha]^{25}_D$ −94 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.10), 217 (4.16), 223 (4.15), 255 (sh) (4.14), 279 (4.29) nm; ^{1}H and ^{13}C NMR, Table S2, Supporting Information; HRESIMS *m*/*z* 405.2035 [M + Na]$^+$ (calcd for $C_{24}H_{30}NaO_4^+$, 405.2036).

6β-Cinnamoyloxy-1β,3β-dihydroxyeudesm-4-ene ***(6):*** white, amorphous solid; $[\alpha]^{25}_D$ −80 (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.25), 216 (4.15), 222 (4.04), 277 (4.25) nm; ^{1}H and ^{13}C NMR, Table S2, Supporting Information; HRESIMS *m*/*z* 407.2191 [M + Na]$^+$ (calcd for $C_{24}H_{32}NaO_4^+$, 407.2193).

6β-Cinnamoyloxy-1β-hydroxyeudesm-3-ene ***(7):*** colorless oil; $[\alpha]^{25}_D$ 34 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.23), 217 (4.20), 222 (4.12), 277 (4.38) nm; ^{1}H and ^{13}C NMR, Table S2, Supporting Information; HRESIMS *m*/*z* 369.2425 [M + H]$^+$ (calcd for $C_{24}H_{33}O_3^+$, 369.2424).

7-epi-6α-Cinnamoyloxy-1β,4α-dihydroxyeudesmane ***(8):*** pale yellow oil; $[\alpha]^{25}_D$ −13 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.10), 217 (4.11), 223 (4.06), 280 (4.27) nm; ^{1}H and ^{13}C NMR, see Table S2, Supporting Information; HRESIMS *m*/*z* 387.2510 [M + H]$^+$ (calcd for $C_{24}H_{35}O_4^+$, 387.2530).

6β-Cinnamoyloxy-4β,9β,15-trihydroxyeudesmane ***(9):*** pale yellow oil; $[\alpha]^{25}_D$ 2 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.06), 210 (4.00), 216 (4.04), 222 (3.98), 276 (4.15) nm; ^{1}H and ^{13}C NMR, Table 1; HRESIMS *m*/*z* 425.2297 [M + Na]$^+$ (calcd for $C_{24}H_{34}NaO_5^+$, 425.2299).

6β-Cinnamoyloxy-1β,3α-dihydroxyeudesm-4(15)-ene ***(10):*** pale yellow, amorphous solid; $[\alpha]^{25}_D$ 29 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.11), 217 (4.12), 223 (4.06), 279 (4.28) nm; ^{1}H and ^{13}C NMR, Table 1; HRESIMS *m*/*z* 407.2192 [M + Na]$^+$ (calcd for $C_{24}H_{32}NaO_4^+$, 407.2193).

6β-Cinnamoyloxy-1β,2α-dihydroxyeudesm-4(15)-ene ***(11):*** yellow oil; $[\alpha]^{25}_D$ 76 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.15), 217 (4.14), 223 (4.08), 278 (4.30) nm; ^{1}H and ^{13}C NMR, Table 1; HRESIMS *m*/*z* 385.2373 [M + H]$^+$ (calcd for $C_{24}H_{33}O_4^+$, 385.2373).

6β-Cinnamoyloxy-1β-hydroxyeudesm-4-ene ***(12):*** colorless oil; $[\alpha]^{25}_D$ 1 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.20), 216 (4.11), 222 (4.02), 278 (4.22) nm; ECD (MeOH, $c = 5.4 \times 10^{-4}$ M, 1 mm path length) λ_{max} ($\Delta\varepsilon$) 207 (−0.68), 223 (+3.08), 231 (+1.22), 237 (+1.23), 279 (−4.18), 312 (+0.16) nm; ^{1}H and ^{13}C NMR, Table 1; HRESIMS *m*/*z* 369.2427 [M + H]$^+$ (calcd for $C_{24}H_{33}O_3^+$, 369.2424).

6β-Cinnamoyloxy-1β,15-dihydroxyeudesm-4-en-3-one ***(13):*** white solid; $[\alpha]^{25}_D$ −115 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.17), 218 (4.23), 223 (4.22), 255 (sh) (4.10), 280 (4.42) nm; ^{1}H and ^{13}C NMR, Table 2; HRESIMS *m*/*z* 421.1984 [M + Na]$^+$ (calcd for $C_{24}H_{30}NaO_5^+$, 421.1986).

6β-Cinnamoyloxy-1β,3α-dihydroxyeudesm-4-ene ***(14):*** yellow oil; $[\alpha]^{25}_D$ −23 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.19), 217 (4.07), 222 (4.00), 277 (4.21) nm; ^{1}H and ^{13}C NMR, Table 2; HRESIMS *m*/*z* 385.2361 [M + H]$^+$ (calcd for $C_{24}H_{33}O_4^+$, 385.2373).

6β-Cinnamoyloxy-1β,15-dihydroxyeudesm-3-ene ***(15):*** yellow oil; $[\alpha]^{25}_D$ 14 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 203 (sh) (4.14), 217 (4.10), 223 (4.03), 279 (4.21) nm; ^{1}H and ^{13}C NMR, Table 2; HRESIMS *m*/*z* 407.2192 [M + Na]$^+$ (calcd for $C_{24}H_{32}NaO_4^+$, 407.2193).

15-Cinnamoyloxy-1β,4β,6β-trihydroxyeudesmane ***(16):*** yellow solid; $[\alpha]^{25}_D$ −12 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.15), 217 (4.17), 222 (4.11), 278 (4.31) nm; ^{1}H and ^{13}C NMR, Table 2; HRESIMS *m*/*z* 425.2295 [M + Na]$^+$ (calcd for $C_{24}H_{34}NaO_5^+$, 423.2299).

Computational Methods. Conformational analysis of compound **12** was performed with MacroModel 9.8 software (Schrödinger LLC) employing the OPLS 2005 (Optimized Potential for Liquid Simulations) force field in H_2O. Conformers within a 2 kcal/mol energy window from the global minimum were selected for geometrical optimization and energy calculation using density function theory (DFT) with Becke's nonlocal three-parameter exchange and

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correlation functional and the Lee−Yang−Parr correlation functional level (B3LYP) using the B3LYP/6-31G** basis set in the gas phase with the Gaussian 09 program package.[38] Vibrational evaluation was done at the same level to confirm minima. Excitation energy (denoted by wavelength in nm), rotatory strength dipole velocity (R_{vel}), and dipole length (R_{len}) were calculated in MeOH by TD-DFT/B3LYP/6-31G**, using the SCRF method, with the CPCM model. ECD curves were obtained on the basis of rotatory strengths with a half-band of 0.24 eV and UV shift using SpecDis v1.64.[39] ECD spectra were calculated from the spectra of individual conformers according to their contribution calculated by Boltzmann weighting.

In Vitro Antifungal Bioassays. Fractions A−Z (26 fractions) obtained from open column chromatography were dissolved at a concentration of 5 mg/mL in DMSO. A 7.5 μL amount of the stock solutions was added to 96-well plates containing 117.5 μL of mineral water and were then serially diluted in the test plate 1:5 and 1:25 with mineral water. Next, 20 μL of a continuously stirred sporangia suspension of *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni ((1.8−2.5) × 10^5 sporangia/mL) (prepared as described below in the subsection "In Vivo Assays on Seedlings") was added to each well. The resulting test concentrations were 250, 50, and 10 μg/mL, respectively.

For the determination of MIC_{100}, defined as the concentration needed to completely inhibit the activity of zoospores, the crude extract and pure compounds **2**, **4**, **5**, **7**−**10**, **14**, and **16** were dissolved in DMSO at a concentration of 2 mg/mL. Compounds **1**, **3**, **6**, **11**−**13**, and **15** were not tested due to the insufficient amounts available. Each solution was then serially diluted 1:1 in 50% DMSO to 3.9 μg/mL. Aliquots of 6 μL of each concentration were added to 94 μL of mineral water before adding 20 μL of the sporangia solution ((1.8−2.5) × 10^5 sporangia/mL). Resulting concentrations of the test products were between 0.195 and 100 μg/mL.

In all in vitro experimental sets, the effect of the solvent (DMSO) alone was tested in at least two replicates in all relevant concentrations.

The activity of zoospores was assessed 2−3 h after setup of the experiment using a binocular at magnifications of 50- to 100-fold. For determination of MIC_{100}, a distinction was made between "no zoospores germinated, or all zoospores inactive" and "active zoospores present". For assessment of activity of fractions and microfractions, inhibition levels were scored as follows: 0, similar to water control; 1, distinct reduction in number and/or activity of zoospores; 2, no zoospores germinated, or all zoospores inactive. To visualize overall inhibitory activity, inhibition levels of all three concentrations were summed up, resulting in values between 0 (no inhibition at highest tested concentrations) and 6 (complete inhibition down to lowest tested concentration).

To calculate mean MIC_{100}, data were $\log_2$ transformed. Data were then retransformed to linear scale.

In Vivo Assays on Seedlings. Bioassays were carried out under semicontrolled conditions in experimental facilities (greenhouse and growth chambers). Small grapevine (*Vitis vinifera* L.) cv. "Chasselas" seedlings were transplanted to individual pots (0.275 L) containing a standard substrate ("Einheitserde Typ 0", Gebr. Patzer GmbH & Co. KG) previously amended with 3 g/L of a mineral fertilizer (Tardit 3M, Hauert Günther Düngerwerke GmbH). Plants were grown in the greenhouse at a minimal temperature of 18 °C under natural light. The photoperiod was extended with sodium high-pressure lamps to 16 h. Plants were used for bioassays when they had three or four fully developed leaves (2−3 weeks after transplanting).

Each experimental set included a nontreated noninoculated control, a water-treated inoculated control, and a standard treatment (copper hydroxide, Kocide Opti, DuPont de Nemours) at two concentrations (300 and 30 μg/mL of copper). All experiments included six replicate plants per treatment. A formulation of *V. lanata* extract was used, containing 5% of the extract, 84% of a solvent (ethyl acetate), and 11% of an emulsifier (Emulsogen EL360, Clariant) in order to enhance solubility in water. The formulation was added to demineralized water at concentrations of 1, 0.5, 0.25, and 0.125 mg plant extract/mL. As a control, a blank formulation was tested at corresponding concentrations. Plants were sprayed with the test products using an air-assisted hand sprayer (DeVilbiss Compact MINI HVLP Touch-Up spray gun) until the leaves (adaxial and abaxial side) were completely covered with a dense layer of small droplets. Plants were left subsequently to dry at room temperature before inoculation. *P. viticola* sporangia suspensions were prepared from previously infected plants by washing freshly sporulating grapevine leaves with water and filtering through cheese cloth. The concentration of the sporangia suspensions was adjusted to 5 × 10^4 sporangia/mL. Plants were spray-inoculated using the air-assisted hand sprayer on the abaxial leaf side. Inoculated plants were subsequently incubated at 20−21 °C and 80−99% relative humidity (RH) in the light for 24 h. Then, plants were maintained at 20 °C, 60−80% RH, with a 16/8-h day/night light regime. Next, 5 to 6 days after inoculation, plants were incubated overnight in the dark at 20 °C and 80−99% RH to promote sporulation. Disease incidence (the percentage of leaves with disease symptoms) and disease severity (the percentage of leaf area covered by lesions) were assessed 6 to 7 days after inoculation. All disease assessments were made using continuous values of percentage based on the European and Mediterranean Plant Protection Organization (EPPO) standard scale.[40]

Efficacies were calculated according to Abbott[41] as $(1 - (A \times B^{-1})) \times 100$, with A = disease severity on an individual plant and B = mean disease severity of control plants.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00868.

HPLC-activity profiling of active fractions (Frs P, Q, U, W, and X); ^{1}H and ^{13}C NMR spectroscopic data for compounds **1**−**8**; ECD spectra of compounds **1**−**16**; 1D selective NOESY of compound **6**; key COSY, HMBC, and NOESY correlations of compounds **10**−**15**; UV spectrum of compound **12** in MeOH; and 1D and 2D NMR spectra of compounds **9**−**16** (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +41-61-2071534. Fax: +41-61-2071474. E-mail: olivier.potterat@unibas.ch.

ORCID

Matthias Hamburger: 0000-0001-9331-273X

Olivier Potterat: 0000-0001-5962-6516

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Agrios, G. N. *Plant Pathology*, 5th ed.; Academic Press: San Diego, CA, USA, 2005; pp 428−433.

(2) Speiser, B.; Mieves, E.; Tamm, L. *Agrarforsch. Schweiz* **2015**, *6*, 160−165.

(3) Borkow, G.; Gabbay. *Curr. Med. Chem.* **2005**, *12*, 2163−2175.

DOI: 10.1021/acs.jnatprod.7b00868
J. Nat. Prod. 2017, 80, 3296−3304

(4) Dagostin, S.; Schärer, H.-J.; Pertot, I.; Tamm, L. *Crop Prot.* **2011**, *30*, 776−788.
(5) Gitzel, J.; Kühne, S. *J. Kult.pflanzen* **2016**, *68*, 125−129.
(6) Kienzle, J. In *Alternativen zur Anwendung von Kupfer als Pflanzschutzmittel*. 7. Fachgespräch am 6. Juni 2002 in Berlin-Dahlem. Berichte aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft, 2003; Vol. *118*, pp 19−20.
(7) Paoletti, M. G.; Sommaggio, D.; Favretto, M. R.; Petruzzelli, G.; Pezzarossa, B.; Barbafieri, M. *Appl. Soil Ecol.* **1998**, *10*, 137−150.
(8) Kula, C.; Guske, S. In *Alternativen zur Anwendung von Kupfer als Pflanzschutzmittel*. 7. Fachgespräch am 6. Juni 2002 in Berlin-Dahlem. Berichte aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft, 2003; Vol. *118*, pp 11−16.
(9) Kula, H. In *Alternativen zur Anwendung von Kupfer als Pflanzschutzmittel*. 7. Fachgespräch am 6. Juni 2002 in Berlin-Dahlem. Berichte aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft, 2003; Vol. *118*, pp 6−10.
(10) Eijsackers, H.; Beneke, P.; Maboeta, M.; Louw, J. P. E.; Reinecke, A. J. *Ecotoxicol. Environ. Saf.* **2005**, *62*, 99−111.
(11) Ntalli, N. G.; Menkissoglu-Spiroudi, U. In *Pesticides - Formulations, Effects, Fate*; Stoytcheva, M., Ed.; InTech: Rijeka, Croatia, 2011; Chapter 1, pp 3−24.
(12) Dang, Q. L.; Lim, C.-H.; Kim, J.-C. *Sigmulbyeong Yeon'gu* **2012**, *18*, 175−185.
(13) Barkai-Golan, R. In *Postharvest Diseases of Fruits and Vegetables*; Elsevier: Amsterdam, The Netherlands, 2001; Chapter 9, pp 147−188.
(14) Schnee, S.; Queiroz, E. F.; Voinesco, F.; Marcourt, L.; Dubuis, P.-H.; Wolfender, J.-L.; Gindro, K. *J. Agric. Food Chem.* **2013**, *61*, 5459−5467.
(15) Castillo, F.; Hernández, D.; Gallegos, G.; Rodríguez, R.; Aguilar, C. N. In *Fungicides for Plant and Animal Diseases*; Dhanasekaran, D., Ed.; InTech: Rijeka, Croatia, 2012; pp 91−106.
(16) Dagostin, S.; Formolo, T.; Giovannini, O.; Pertot, I.; Schmitt, A. *Plant Dis.* **2010**, *94*, 575−580.
(17) Cohen, Y.; Wang, W.; Ben-Daniel, B.-H.; Ben-Daniel, Y. *Phytopathology* **2006**, *96*, 417−424.
(18) Scherf, A.; Treutwein, J.; Kleeberg, H.; Schmitt, A. *Eur. J. Plant Pathol.* **2012**, *134*, 755−762.
(19) Mulholland, D. A.; Thuerig, B.; Langat, M. K.; Tamm, L.; Nawrot, D. A.; James, E. E.; Qayyum, M.; Shen, D.; Heap, K.; Jones, A.; Hokkanen, H.; Demidova, N.; Izotov, D.; Schärer, H.-J. *Crop Prot.* **2017**, *102*, 104−109.
(20) Thuerig, B.; Ramseyer, J.; Hamburger, M.; Oberhänsli, T.; Potterat, O.; Schärer, H.-J.; Tamm, L. *Pest Manage. Sci.* **2016**, *72*, 1718−1726.
(21) Potterat, O.; Hamburger, M. *Planta Med.* **2014**, *80*, 1171−1181.
(22) Tropicos, *Verbesina lanata*, http://tropicos.org (accessed on June 06, 2017).
(23) Mora, F. D.; Alpan, L.; McCracken, V. J.; Nieto, M. *Nat. Prod. J.* **2013**, *3*, 140−150.
(24) Mabberley, D. J. *Mabberley's Plant-book: A Portable Dictionary of Plants, Their Classification and Uses*; Cambridge University Press: Cambridge, UK, 2008; p 895.
(25) Bohlmann, F.; Grenz, M.; Gupta, R. K.; Dhar, A. K.; Ahmed, M.; King, R. M.; Robinson, H. *Phytochemistry* **1980**, *19*, 2391−2397.
(26) Jakupovic, J.; Ellmauerer, E.; Jia, Y.; Bohlmann, F.; Dominguez, X. A.; Hirschmann, G. S. *Planta Med.* **1987**, *53*, 39−42.
(27) Tiansheng, L.; Vargas, D.; Fischer, N. H. *Phytochemistry* **1993**, *34*, 737−742.
(28) Du, K.; De Mieri, M.; Neuburger, M.; Zietsman, P. C.; Marston, A.; van Vuuren, S. F.; Ferreira, D.; Hamburger, M.; van der Westhuizen, J. H. *J. Nat. Prod.* **2015**, *78*, 2494−2504.
(29) Bruno-Colmenárez, J.; Amaro-Luis, J. M.; Ramírez, I.; Delgado-Méndez, P.; Díaz de Delgado, G. *J. Chem. Crystallogr.* **2010**, *40*, 110−115.
(30) Jain, S. C.; Purohit, M.; Sharma, R. *Phytother. Res.* **1988**, *2*, 146−148.
(31) Abad, M. J.; Ansuategui, M.; Bermejo, P. *Arkivoc* **2007**, *7*, 6−145.
(32) Wu, Q.-X.; Shi, Y.-P.; Jia, Z.-J. *Nat. Prod. Rep.* **2006**, *23*, 699−734.
(33) Maatooq, G. T.; Stumpf, D. K.; Hoffmann, J. J.; Hutter, L. K.; Timmermann, B. N. *Phytochemistry* **1996**, *41*, 519−524.
(34) Alarif, W. M.; Al-Footy, K. O.; Zubair, M. S.; Halid Ph, M.; Ghandourah, M. A.; Basaif, S. A.; Al-Lihaibi, S. S.; Ayyad, S.-E. N.; Badria, F. A. *Nat. Prod. Res.* **2016**, *30*, 1150−1155.
(35) Al-Dabbas, M. M.; Hashinaga, F.; Abdelgaleil, S. A.; Suganuma, T.; Akiyama, K.; Hayashi, H. *J. Ethnopharmacol.* **2005**, *97*, 237−240.
(36) Jiang, Z. D. U.S. Patent 5,917,084, 1999.
(37) Thuerig, B.; James, E. E.; Schärer, H.-J.; Langat, M. K.; Mulholland, D. A.; Treutwein, J.; Kleeberg, I.; Ludwig, M.; Jayarajah, P.; Giovannini, O.; Markellou, E.; Tamm, L. *Pest Manage. Sci.* **2017**, DOI: 10.1002/ps.4733.
(38) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. A.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, H. P.; Izmaylov, A. F.; Bloino, J.; Zheng, G.; Sonnenberg, J. L.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery, J. A., Jr.; Peralta, J. E.; Ogliaro, F.; Bearpark, M. J.; Heyd, J.; Brothers, E. N.; Kudin, K. N.; Staroverov, V. N.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A. P.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, N. J.; Klene, M.; Knox, J. E.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Zakrzewski, V. G.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A. D.; Farkas, O.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J. *Gaussian 09*; Gaussian, Inc.: Wallingford, CT, USA, 2009.
(39) Bruhn, T.; Hemberger, A. S. Y.; Bringmann, G. *SpecDis* version 1.64; University of Wuerzburg: Germany, 2015.
(40) European and Mediterranean Plant Protection Organization. *Bull. OEPP* **2001**, *31*, 313−317.
(41) Abbott, W. S. *J. Econ. Entomol.* **1925**, *18*, 265−267.

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Addition/Correction

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Correction to Eudesmane Sesquiterpenes from *Verbesina lanata* with Inhibitory Activity against Grapevine Downy Mildew

Justine Ramseyer, Barbara Thuerig, Maria De Mieri, Hans-Jakob Schärer, Thomas Oberhänsli, Mahabir P. Gupta, Lucius Tamm, Matthias Hamburger, and Olivier Potterat*

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Page 3296: The last sentence of the abstract, "The two major compounds, 6β-cinnamoyloxy-4β,9β,15-trihydroxyeudesmane (**9**) and 6β-cinnamoyloxy-1β,15-dihydroxyeudesm-4-en-3-one (**13**), showed MIC_{100} values of 5 μg/mL and 31 μg/mL, respectively", should be changed to "The two major compounds, 6β-cinnamoyloxy-1β-hydroxyeudesm-4-en-3-one (**5**) and 6β-cinnamoyloxy-1β-hydroxyeudesm-3-ene (**7**), showed MIC_{100} values of 5 μg/mL and 31 μg/mL, respectively." The authors greatly apologize for any inconveniences this error may have caused.

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SUPPORTING INFORMATION

Eudesmane Sesquiterpenes from *Verbesina lanata* with Inhibitory Activity against Grapevine Downy Mildew

Justine Ramseyer,[†] Barbara Thuerig,[‡] Maria De Mieri,[†] Hans-Jakob Schärer,[‡] Thomas Oberhänsli,[‡] Mahabir P. Gupta,[§] Lucius Tamm,[‡] Matthias Hamburger,[†] and Olivier Potterat*[,†]

[†]Division of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel, Klingelbergstrasse 50, 4056 Basel, Switzerland

[‡]Research Institute of Organic Agriculture FiBL, Department of Crop Sciences, Ackerstrasse 113, 5070 Frick, Switzerland

§Center for Pharmacognostic Research on Panamanian Flora, Faculty of Pharmacy, University of Panama, Panama City, Republic of Panama

Corresponding Author

*Tel: +41-61-2071534. Fax: +41-61-2071474. E-mail: olivier.potterat@unibas.ch

TABLE OF CONTENT

Table S1. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds **1-4** ($CDCl_3$; 500 MHz for ^{1}H, ^{13}C extracted from ^{1}H-^{13}C 2D inverse detected experiments; δ in ppm)

	1		**2**[a]		**3**		**4**[b]	
position	δ_H, (*J* in Hz)	δ_C, type	δ_H, (*J* in Hz)	δ_C, type	δ_H, (*J* in Hz)	δ_C, type	δ_H, (*J* in Hz)	δ_C, type
1	1.39, m[c], *β*	43.3, CH_2	1.42, m[c]	40.9, CH_2	3.66, m, *α*	74.9, CH	3.72, dd (12.9, 3.0), *α*	73.9, CH
	1.13, m[c], *α*		1.25, m[c]					
2	1.58, m[c]	19.9, CH_2	1.77, dq, (13.4, 3.9), *α*	26.6, CH_2	2.17, br d (13.4), *α*	33.6, CH_2	2.34, br d (14.0), *α*	30.6, CH_2
	1.58, m[c]		1.55 m, *β*		1.80, br t (13.4), *β*		1.76, m[c], *β*	
3	1.64, m[c]	45.3, CH_2	3.46, dd (12.0, 3.9), *α*	78.8, CH	4.44, br s, *β*	87.0, CH	4.36, br d (2.7), *β*	85.0, CH
	1.24, m[c]							
4	-	71.5, C	-	75.2, C	-	142.4, C	-	128.5, C
5	1.42, m[c], *α*	57.0, CH	1.33, d (2.2), *α*	55.1, CH	2.33, br s, *α*	46.9, CH	-	139.9, C
6	5.83, br s, *α*	69.3, CH	5.91, br s, *α*	69.4, CH	5.79, br s, *α*	71.2, CH	6.10, br s, *α*	70.9, CH
7	1.13, m[c], *α*	50.1, CH	1.12, m, *α*	49.7, CH	1.19, m, *α*	50.2, CH	1.09, m[c], *α*	48.7, CH
8	1.63, m[c]	21.1, CH_2	1.66, m[c]	21.1, CH_2	1.70, br d (12.0), *α*	20.2, CH_2	1.73, m[c]	20.3, CH_2
	1.58, m[c]		1.61, m[c]		1.62, br q (12.0), *β*		1.73, m[c]	
9	1.70, m[c]	44.0, CH_2	1.64, m[c]	45.0, CH_2	2.06, br d (12.0), *β*	37.1, CH_2	2.10, dt (13.4, 2.8), *β*	38.3, CH_2
	1.43, m[c]		1.21, m[c]		1.27, m, *α*		1.21, m[c], *α*	
10	-	34.5, C	-	34.7, C	-	40.2, C	-	39.4, C
11	1.44, m[c]	28.7, CH	1.47, m[c]	28.6, CH	1.40, m	28.1, CH	1.59, m	29.1, CH
12	0.92, d (6.7)	21.2, CH_3	0.93, d (6.7)	21.1, CH_3	1.05, br d (6.1)	21.8, CH_3	0.96, d (6.5)	20.8, CH_3
13	0.89, d (6.7)	20.5, CH_3	0.91, d (6.7)	20.6, CH_3	0.89, br d (6.1)	20.2, CH_3	0.94, d (6.5)	20.8, CH_3
14	1.18, s	21.3, CH_3	1.20, br s	21.5, CH_3	1.04, m[c]	12.5, CH_3	1.13, s	17.0, CH_3
15	1.19, s	24.4, CH_3	1.17, s	17.7, CH_3	5.17, br s 5.12, br s	116.2, CH_2	2.07, s	17.5, CH_3
1’	-	167.9, C	-	168.1, C	-	167.0, C	-	166.1, C
2’	6.44, d (16.0)	118.3, CH	6.47, d (15.9)	118.1, CH	6.42, d (15.9)	118.1, CH	6.42, d (16.0)	118.4, CH
3’	7.70, d (16.0)	145.3, CH	7.73, d (15.9)	145.5, CH	7.72, d (15.9)	145.0, CH	7.67, d (16.0)	144.8, CH
4’	-	134.2, C	-	134.3, C	-	134.2, C	-	134.3, C
5’, 9’	7.54, m	128.0, CH	7.56, m	128.1, CH	7.54, m	128.0, CH	7.53, m	128.1, CH
6’, 8’	7.38, m[c]	128.7, CH	7.39, m[c]	128.7, CH	7.39, m[c]	128.6, CH	7.38, m[c]	128.8, CH
7’	7.38, m[c]	130.2, CH	7.40, m[c]	130.3, CH	7.38, m[c]	130.2, CH	7.37, m[c]	130.2, CH

[a] C-14 and C-15 shifts inverted by Jakupovic et al., *Planta Med* **1987**, *53*, 39-42.
[b] C-4 shift reported as 138.3 ppm by Tiansheng et al., *Phytochemistry* **1993**, *34*, 737-742.
[c] Overlapping signals.

Table S2. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds **5-8** ($CDCl_3$; 500 MHz for ^{1}H, ^{13}C extracted from ^{1}H-^{13}C 2D inverse detected experiments; δ in ppm)

	5		**6**		**7**		**8**	
position	δ_H, (*J* in Hz)	δ_C, type	δ_H, (*J* in Hz)	δ_C, type	δ_H, (*J* in Hz)	δ_C, type	δ_H, (*J* in Hz)	δ_C, type
1	3.78, dd (11.6, 6.4), *α*	74.9, CH	3.40, br d (12.0), *α*	75.4, CH	3.53, dd (10.2, 6.3), *α*	76.6, CH	3.30, dd (10.3, 4.0), *α*	78.9, CH
2	2.60, m[a]	42.4, CH_2	2.11, ddd (11.5, 7.0, 3.0), *α*	36.5, CH_2	2.27, m, *α*	32.2, CH_2	1.61, m[a]	27.8, CH_2
	2.60, m[a]		1.74 m[a], *β*		2.00, m[a], *β*		1.53, m[a]	
3	-	198.2, C	4.10, q[b] (7.0), *α*	70.7, CH	5.35, br m	121.2, CH	1.66, m[a]	39.1, CH_2
							1.55, m[a]	
4	-	133.8, C	-	134.9, C	-	133.3, C	-	71.7, C
5	-	154.8, C	-	134.5, C	2.10, br s, *α*	50.9, CH	2.00, br d (1.19), *α*	49.8, CH
6	6.31, d (1.8), *α*	70.5, CH	6.10, br d (2.1), *α*	71.3, CH	5.76, br s, *α*	71.4, CH	5.48, dd (11.9, 4.3), *β*	76.8, CH
7	1.20, m, *α*	48.4, CH	1.05, ddd (12.5, 6.0, 4.0), *α*	48.9, CH	1.21, m[a], *α*	49.3, CH	1.82, m, *β*	44.0, CH
8	1.93-1.78, m	19.9, CH_2	1.76, m[a]	20.5, CH_2	1.72, m[a], *α*	20.3, CH_2	1.67, m[a]	23.0, CH_2
			1.71, m[a]		1.64, qd (13.1, 3.5), *β*		1.63, m[a]	
9	2.21, dt (13.1, 3.4), *β*	37.8, CH_2	1.99, m[a], *β*	38.3, CH_2	2.03, m[a], *β*	35.6, CH_2	1.52, m[a], *β*	35.0, CH_2
	1.36, m[a], *α*		1.17, td (12.7, 5.0), *α*		1.18, m[a], *α*		1.19, td (13.5, 4.0), *α*	
10	-	41.2, C	-	39.6, C	-	37.7, C	-	42.0, C
11	1.65, m	28.8, CH	1.57, m	28.8, CH	1.49, m	28.6, CH	2.00, m[a]	25.3, CH
12	1.00, d (6.7)	20.7, CH_3	0.93, d (6.7)	20.6, CH_3	1.06, d (6.7)	22.0, CH_3	0.93, d (6.7)	23.3, CH_3
13	0.98, d (6.7)	20.6, CH_3	0.95, d (6.7)	20.6, CH_3	0.89, d (6.7)	20.0, CH_3	0.86, d (6.7)	22.1, CH_3
14	1.31, s	16.5, CH_3	1.18, s	18.5, CH_3	1.12, br s	12.2, CH_3	0.95, br s	13.4, CH_3
15	2.03, s	11.0, CH_3	1.99, br s[a]	14.6, CH_3	1.70, br s	20.6, CH_3	1.10, s	24.0, CH_3
1’	-	165.6, C	-	165.9, C	-	166.1, C	-	165.2, C
2’	6.41, d (15.9)	117.6, CH	6.40, d (16.2)	118.5, CH	6.40, d (15.9)	118.6, CH	6.31, d (16.0)	117.4, CH
3’	7.68, d (15.9)	145.3, CH	7.64, d (16.2)	144.3, CH	7.68, d (15.9)	144.5, CH	7.59, d (16.0)	145.8, CH
4’	-	134.2, C	-	134.3, C	-	134.2, C	-	134.0, C
5’, 9’	7.53, m	128.0, CH	7.51, m	127.9, CH	7.52, m	128.0, CH	7.43, m	128.1, CH
6’, 8’	7.38, m[a]	128.7, CH	7.36, m[a]	128.8, CH	7.38, m[a]	128.7, CH	7.29, m[a]	128.7, CH
7’	7.38, m[a]	130.2, CH	7.36, m[a]	130.0, CH	7.37, m[a]	130.0, CH	7.29, m[a]	130.5, CH

[a] Overlapping signals.
[b] Apparent multiplicity.

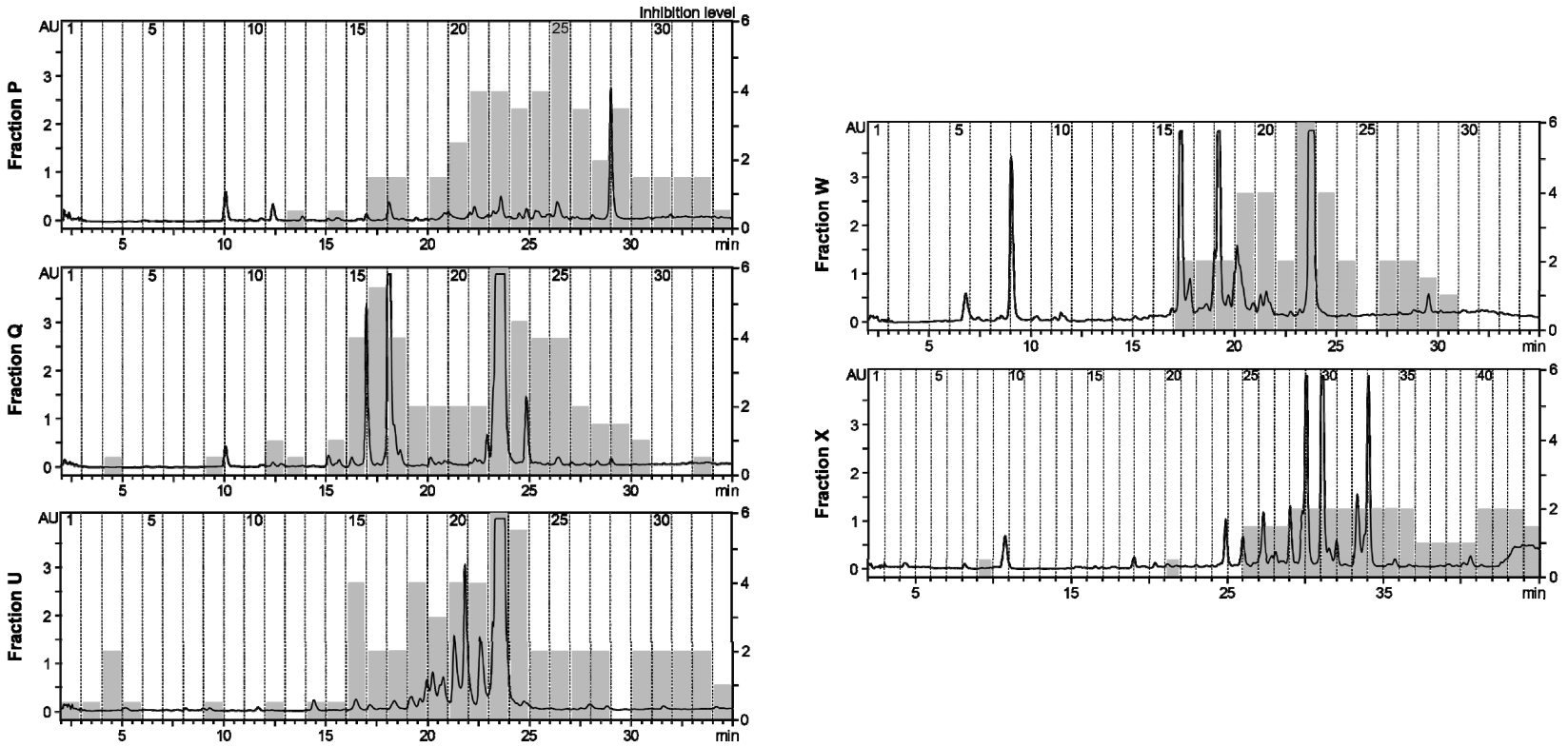


Figure S1. HPLC-profiling of *Verbesina lanata* active fractions (P, Q, U, W, and X) against *Plasmopara viticola*.
Separations were performed on a SunFire C18 column (3.5 µm, 3.0 x 150 mm). The mobile phase consisted of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B); Gradients: Frs P and Q, 40 to 100% B in 30 min; Fr U, 40 to 100% B; Fr W, 30 to 100% B in 30 min; and Fr X, 30 to 70% B in 40 min. Each method was followed by 100% B for 5 min. Flow rate: 0.4 mL/min. Detection at 254 nm. Collection every 60 sec from 2 min to the end. 0.9 mg of each fraction were injected in 3 portions. After drying, micro-fractions were re-dissolved in 50 µL DMSO. 7.5 µl of each fraction were added to 117.5 µL of 'Evian' water and then serially diluted 1:5 and 1:25 in the test plate. 20 µL of a sporangia solution were added to each well. Activity was assessed after 2-3 h using a binocular (magnifications of 50 to 100 fold). Inhibition levels were scored as follows: 0 = similar to water control; 1 = distinct reduction in number and or activity of zoospores; 2 = no zoospores germinated, or all zoospores inactive; Inhibition levels at the three concentrations were summed up, resulting in values between 0 (no inhibition at highest tested concentrations) and 6 (complete inhibition down to lowest tested concentration).

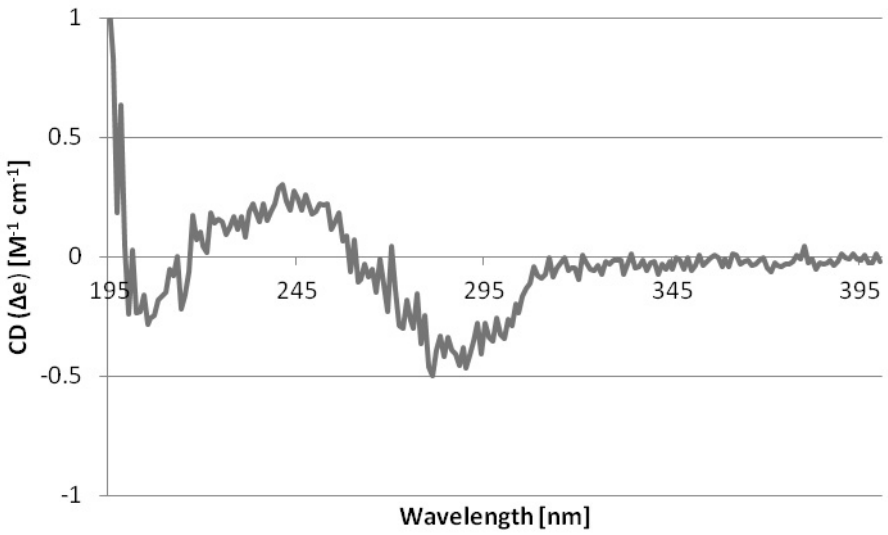


Figure S2. ECD spectrum of compound **1** in MeOH (0.4 mg/mL)

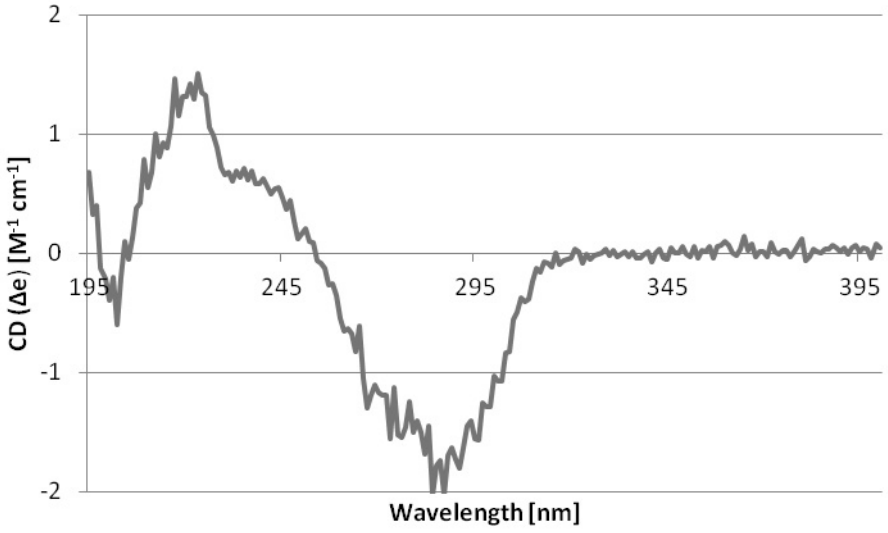


Figure S3. ECD spectrum of compound **2** in MeOH (0.2 mg/mL)

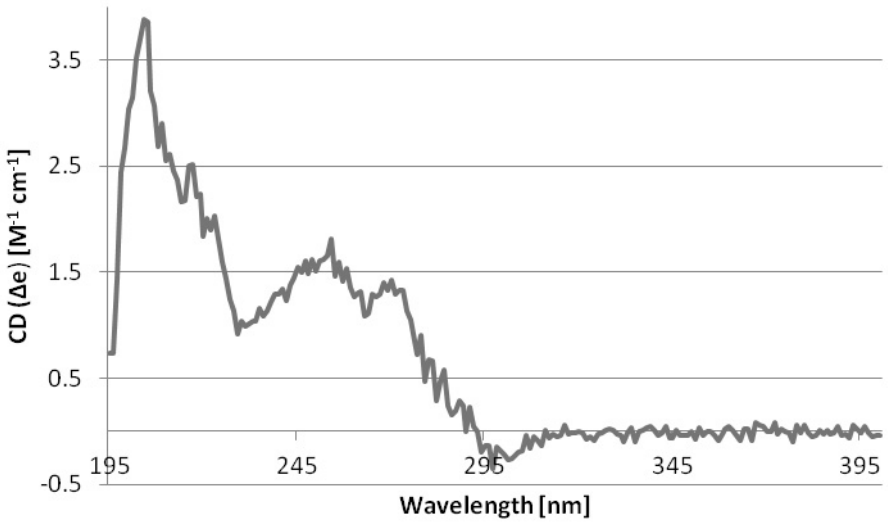


Figure S4. ECD spectrum of compound **3** in MeOH (0.2 mg/mL)

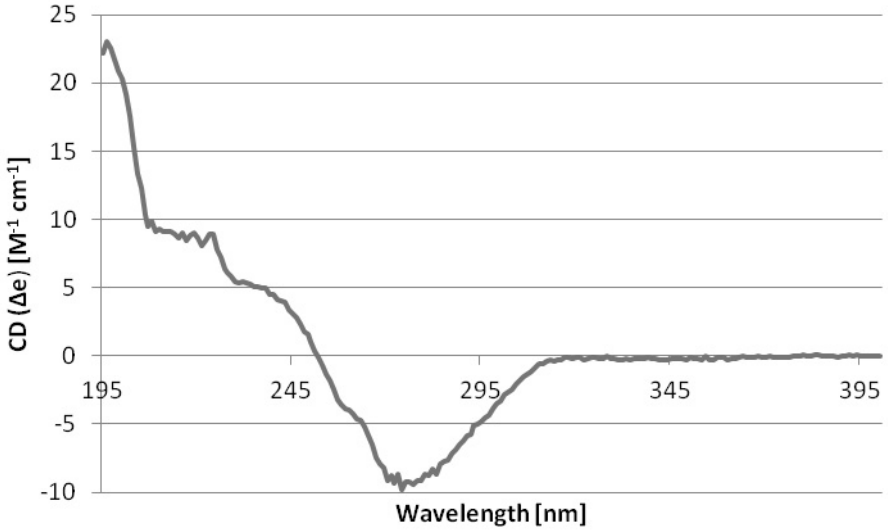


Figure S5. ECD spectrum of compound **4** in MeOH (0.2 mg/mL)

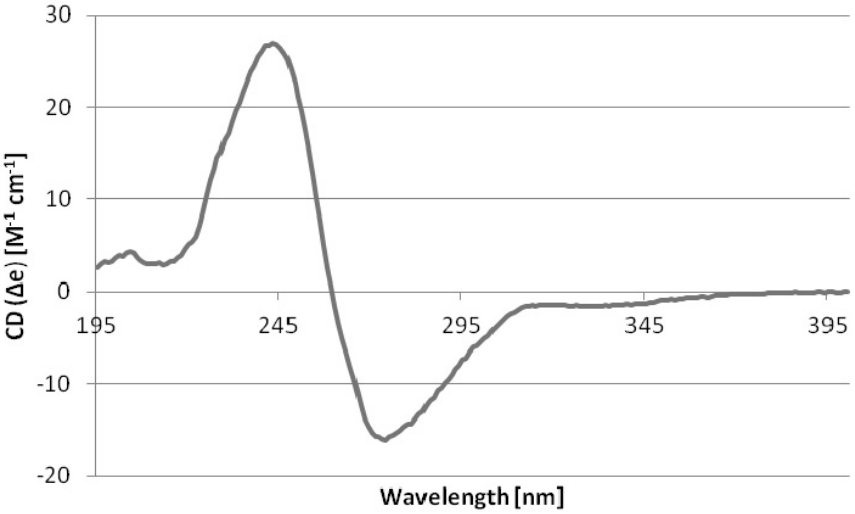


Figure S6. ECD spectrum of compound **5** in MeOH (0.2 mg/mL)

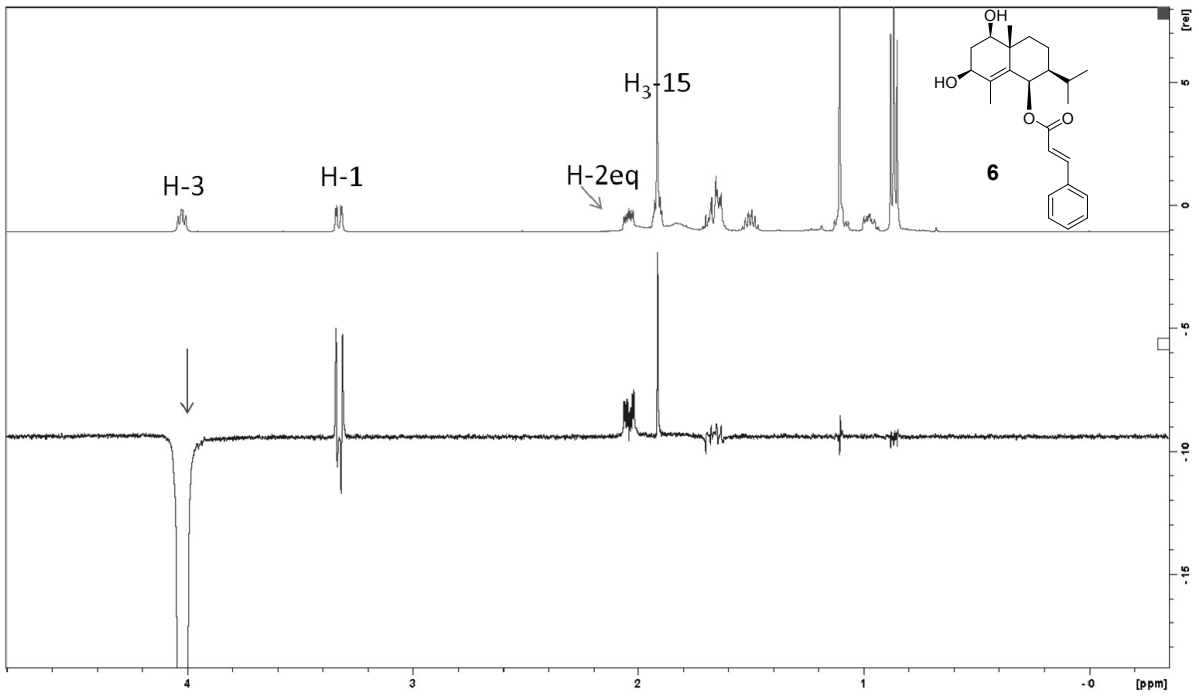


Figure S7. 1D selective NOESY of compound **6** (500 MHz, D8 = 0.5 sec, $CDCl_3$)

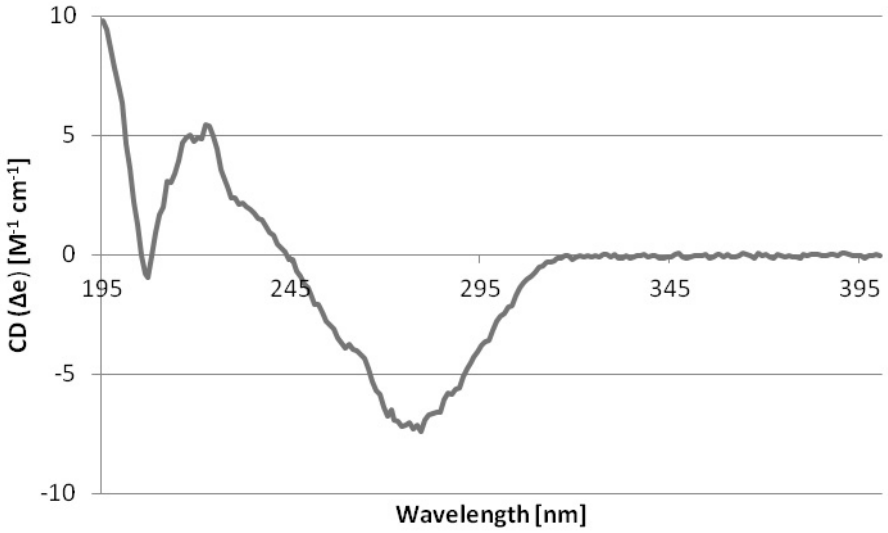


Figure S8. ECD spectrum of compound **6** in MeOH (0.2 mg/mL)

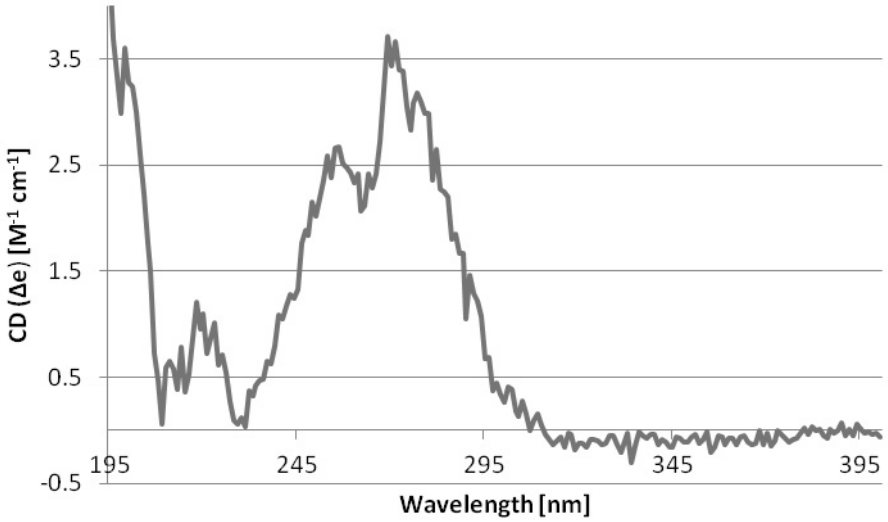


Figure S9. ECD spectrum of compound **7** in MeOH (0.2 mg/mL)

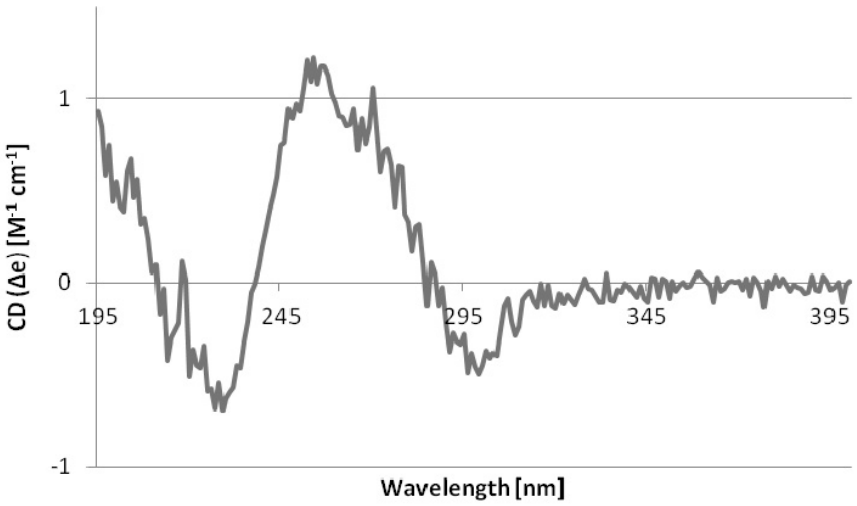


Figure S10. ECD spectrum of compound **8** in MeOH (0.2 mg/mL)

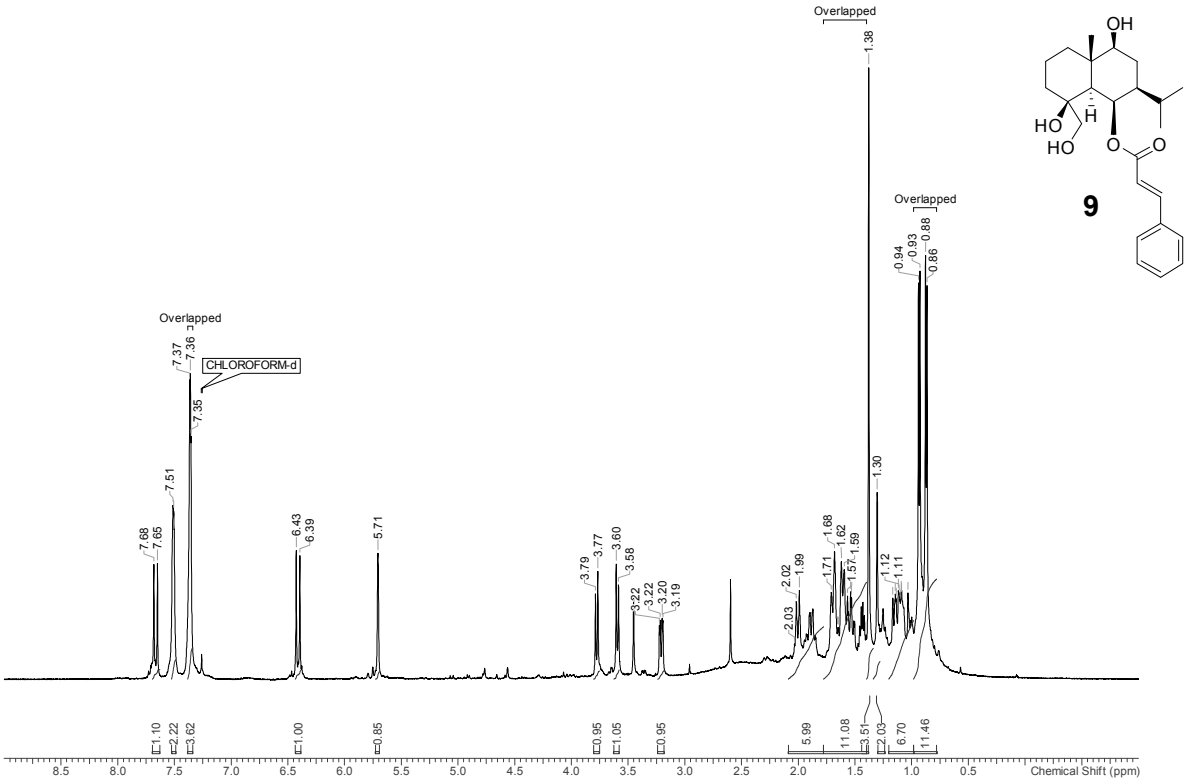


Figure S11. ^{1}H NMR spectrum of compound **9** (500 MHz, $CDCl_3$)

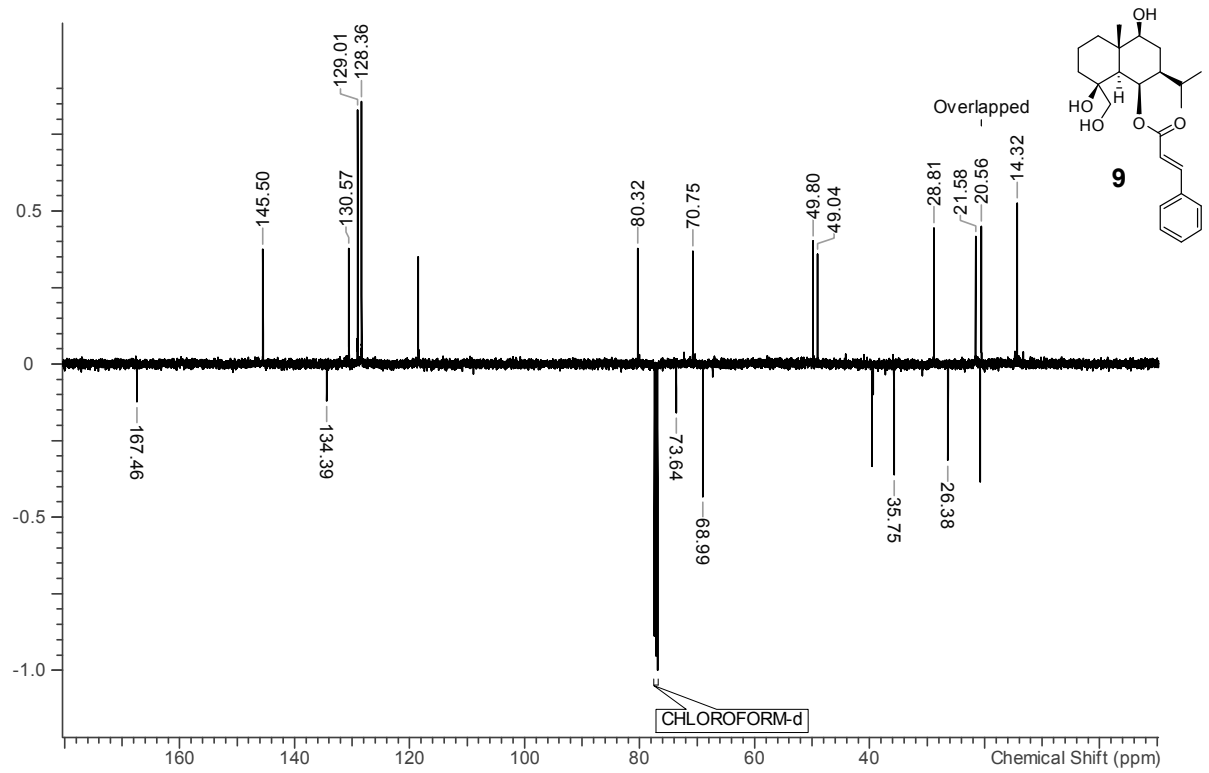


Figure S12. ^{13}C-DEPTq spectrum of compound **9** (125 MHz, $CDCl_3$)

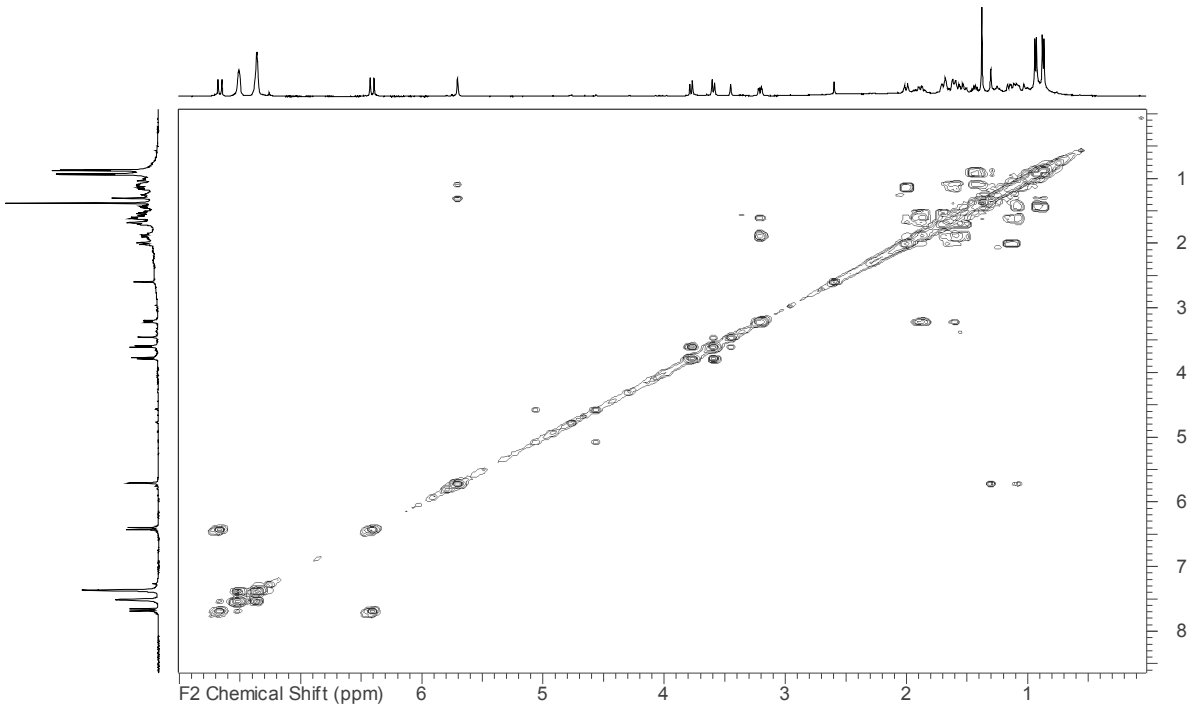


Figure S13. ^{1}H-^{1}H COSY spectrum of compound **9** (500 MHz, $CDCl_3$)

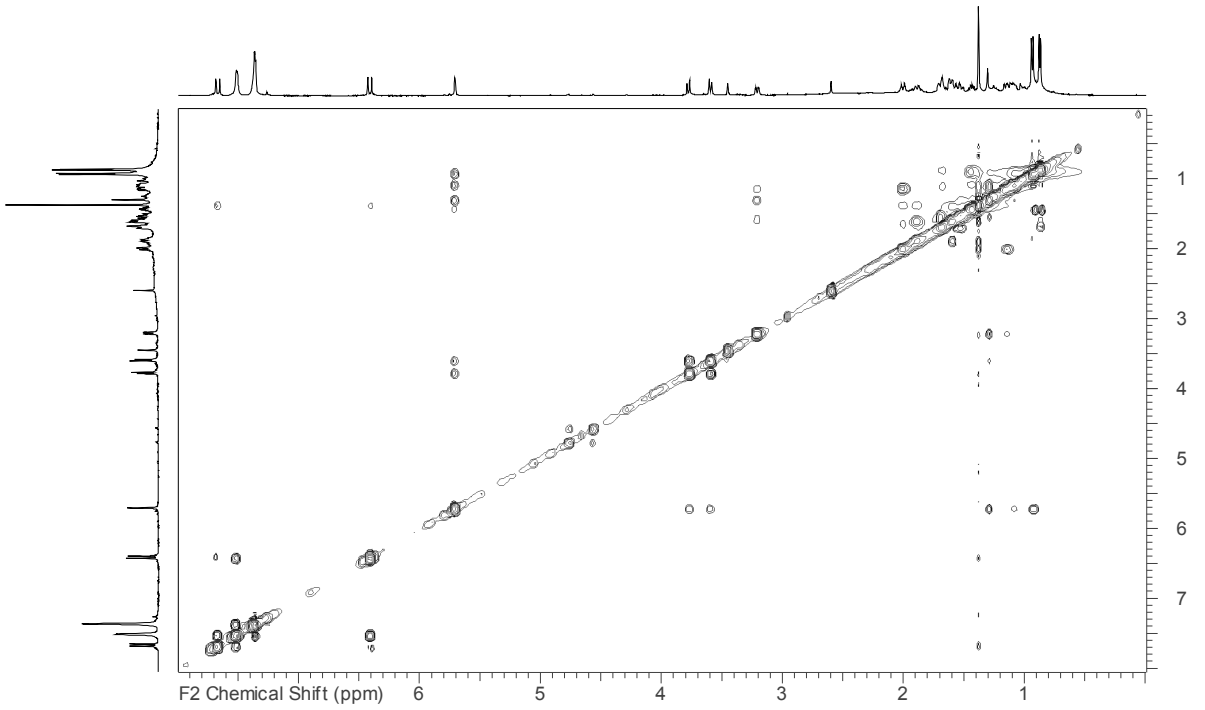


Figure S14. ^{1}H-^{1}H NOESY spectrum of compound **9** (500 MHz, $CDCl_3$)

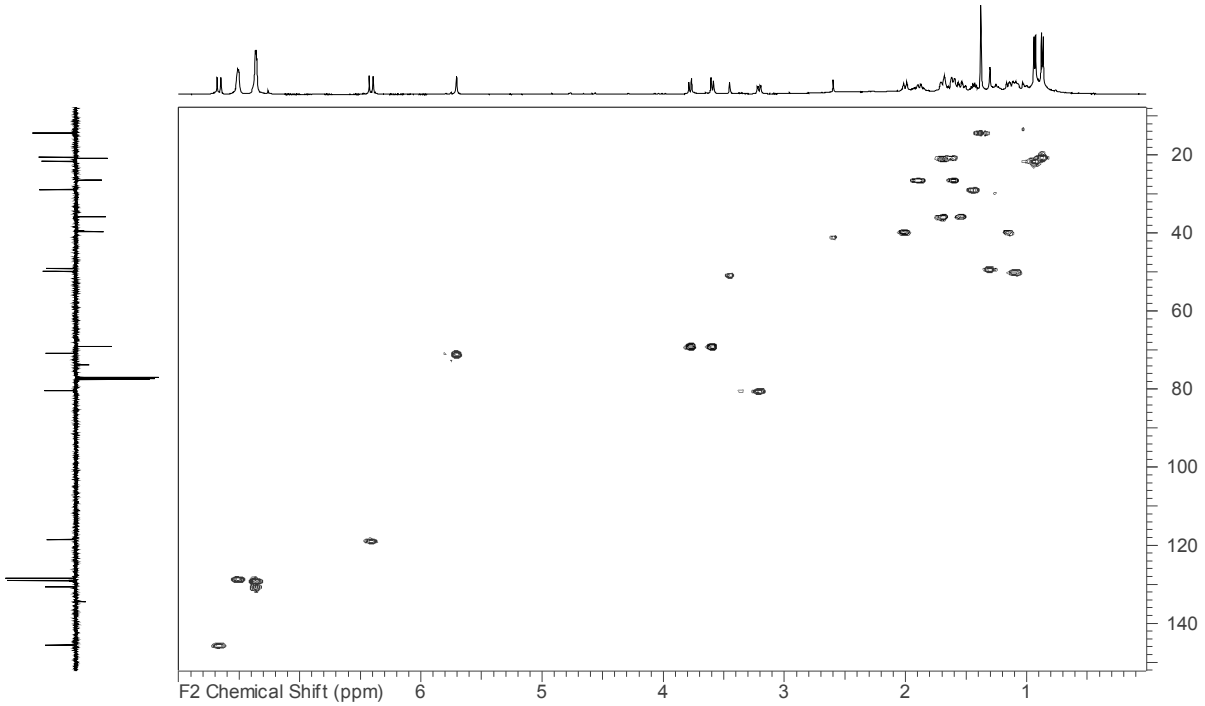


Figure S15. HSQC-DEPT spectrum of compound **9** (500 MHz, $CDCl_3$)

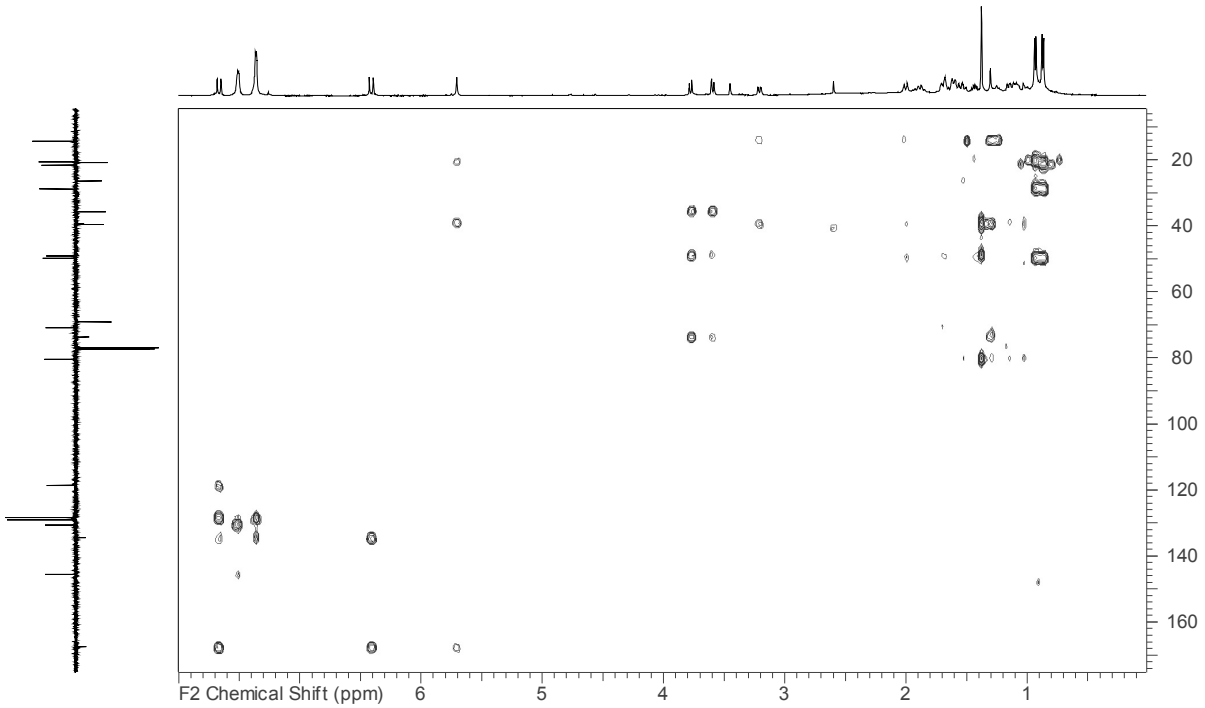


Figure S16. HMBC spectrum of compound **9** (500 MHz, $CDCl_3$)

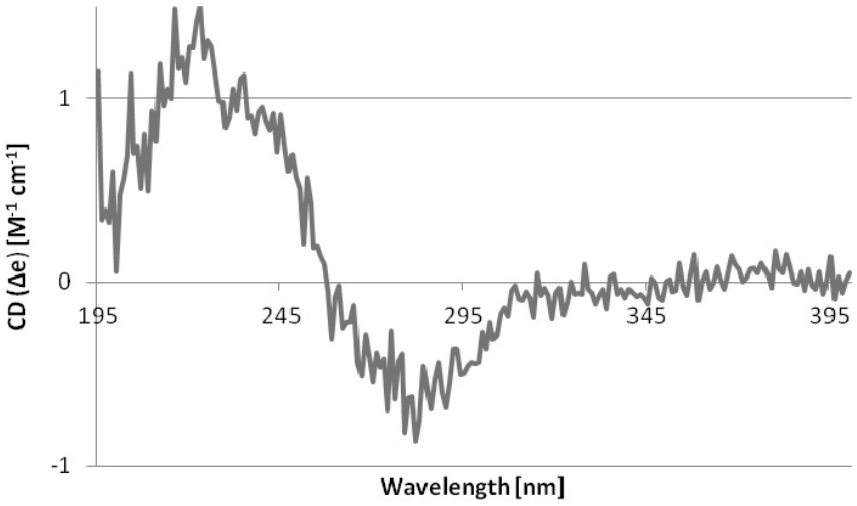


Figure S17. ECD spectrum of compound **9** in MeOH (0.2 mg/mL)

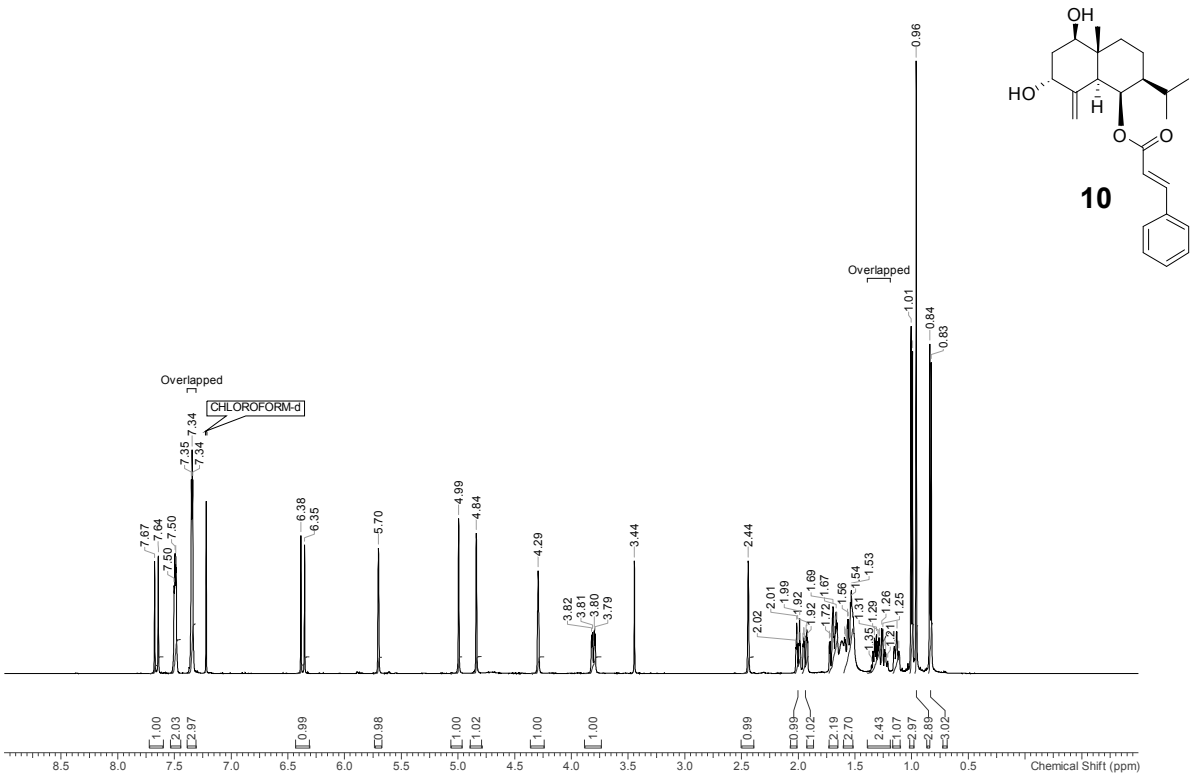


Figure S18. ^{1}H NMR spectrum of compound **10** (500 MHz, $CDCl_3$)

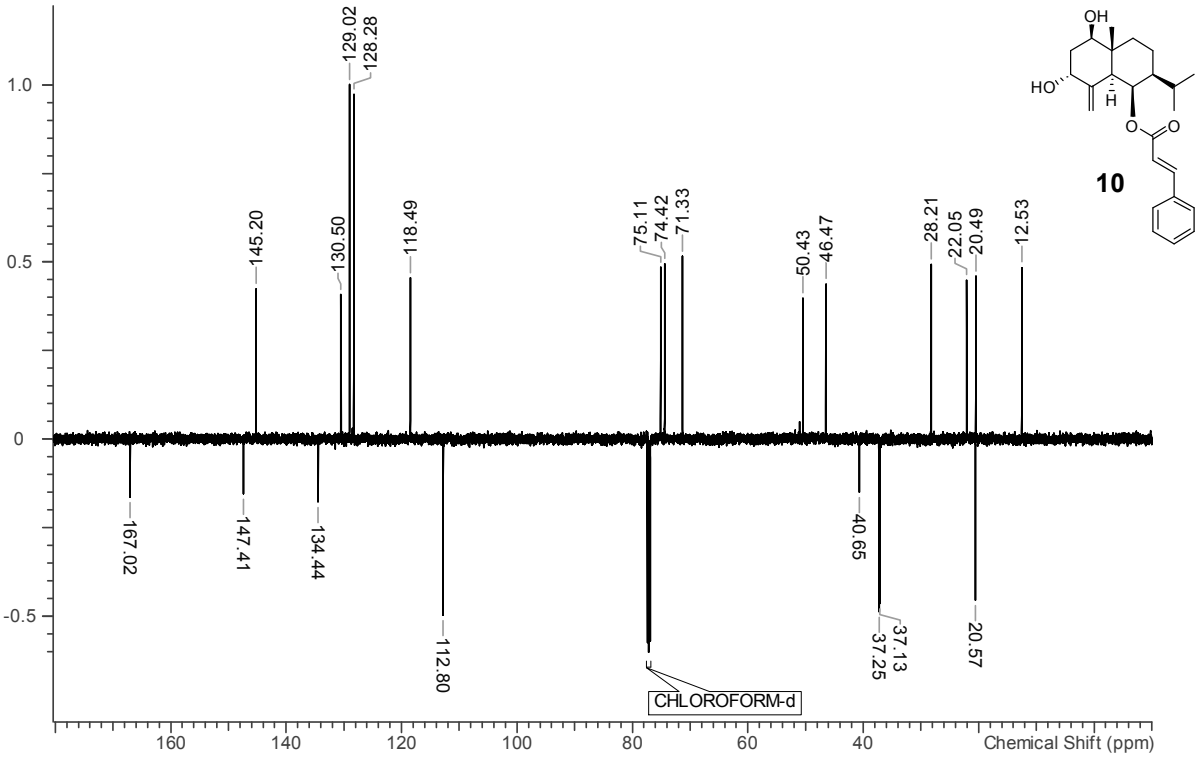


Figure S19. ^{13}C-DEPTq spectrum of compound **10** (125 MHz, $CDCl_3$)

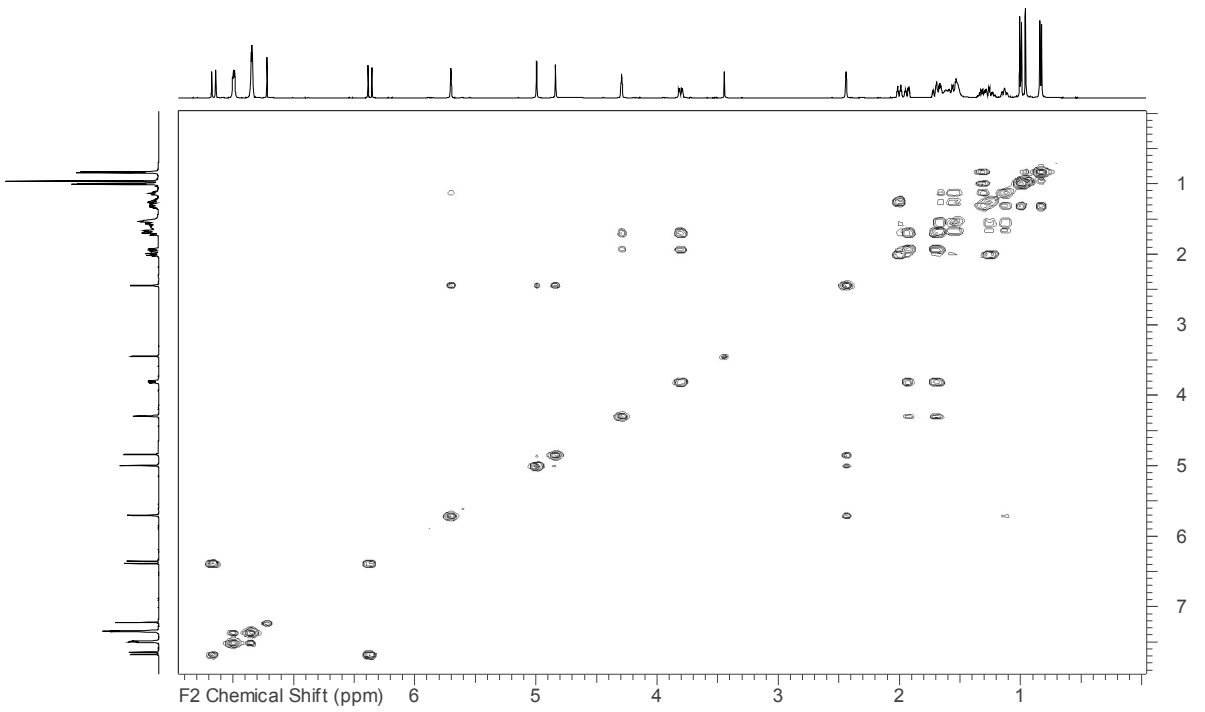


Figure S20. ^{1}H-^{1}H COSY spectrum of compound **10** (500 MHz, $CDCl_3$)

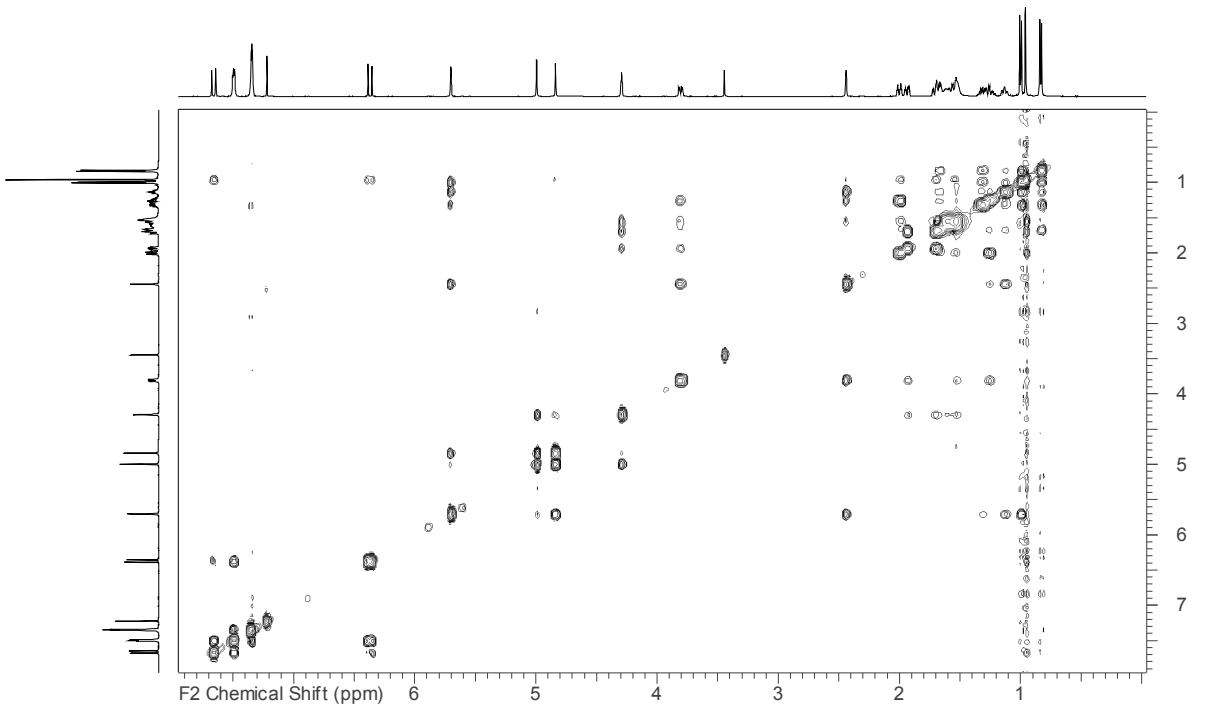


Figure S21. ^{1}H-^{1}H NOESY spectrum of compound **10** (500 MHz, $CDCl_3$)

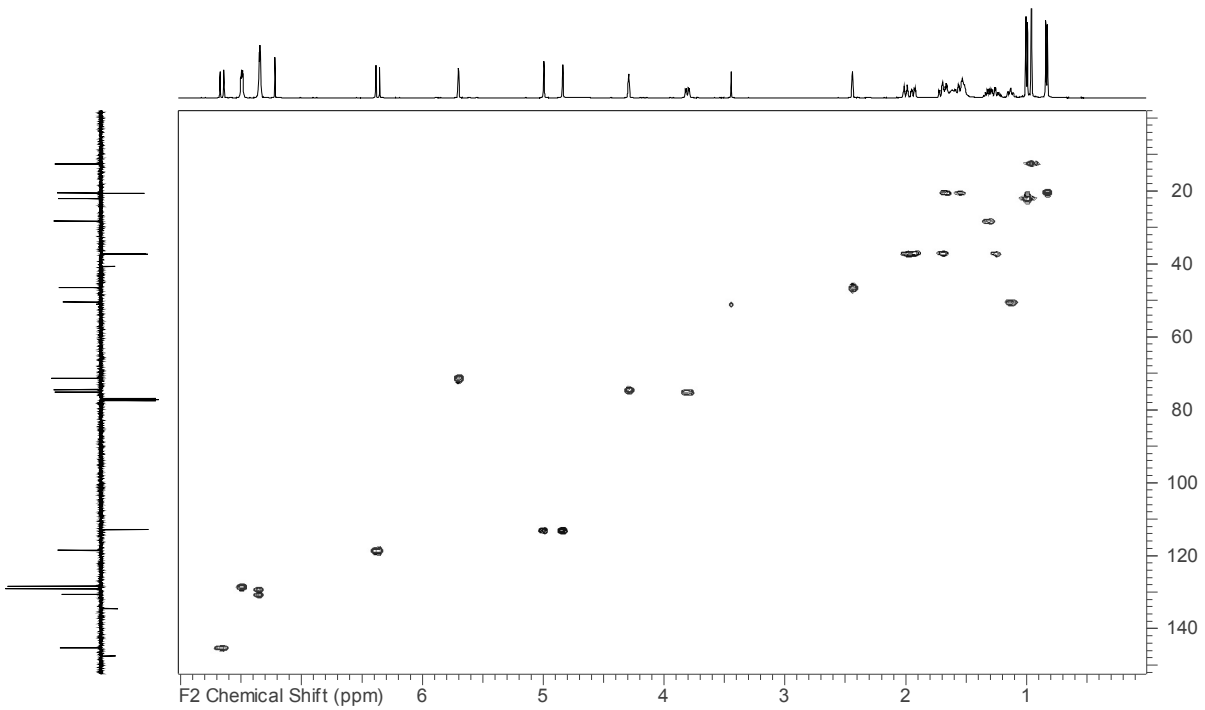


Figure S22. HSQC-DEPT spectrum of compound **10** (500 MHz, $CDCl_3$)

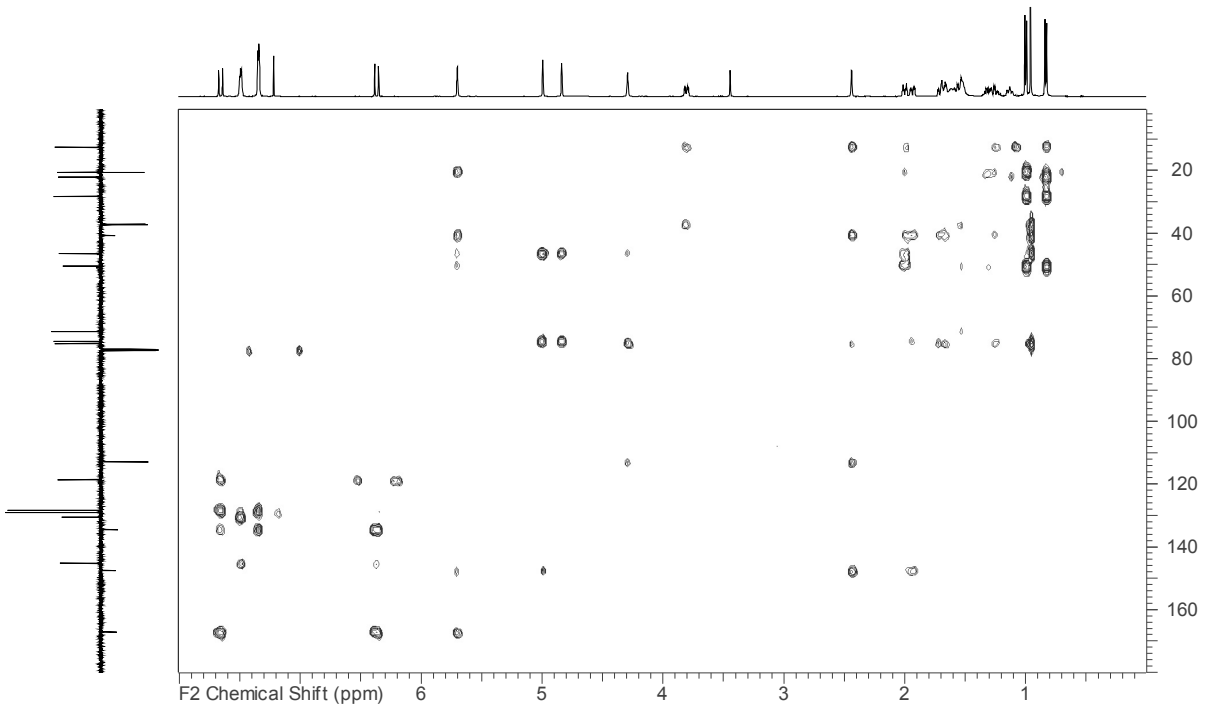


Figure S23. HMBC spectrum of compound **10** (500 MHz, $CDCl_3$)

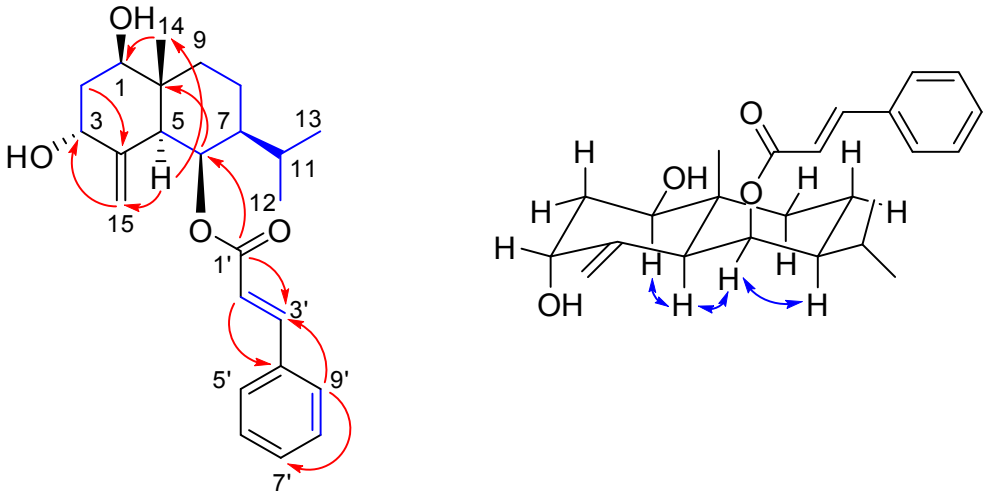


Figure S24. Key COSY (blue bonds), HMBC (red arrows), and NOESY correlations (blue arrows) of compound **10**

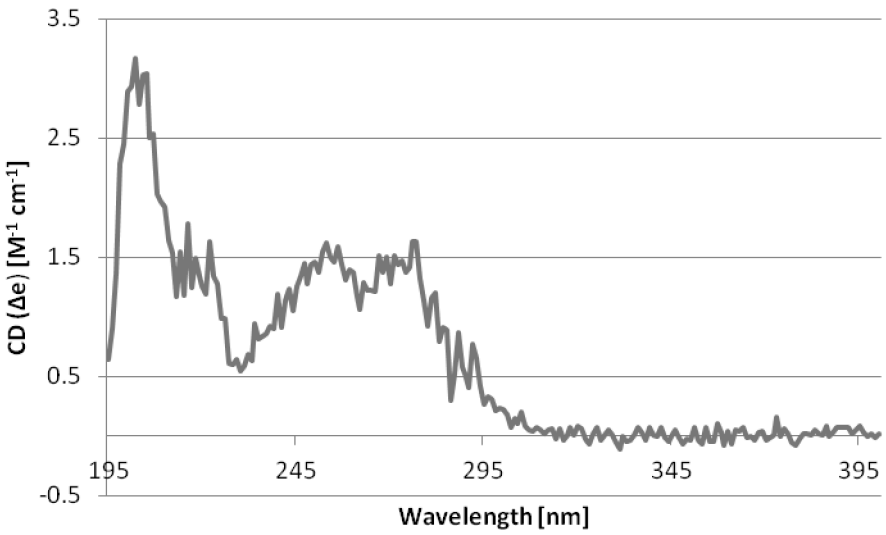


Figure S25. ECD spectrum of compound **10** in MeOH (0.2 mg/mL)

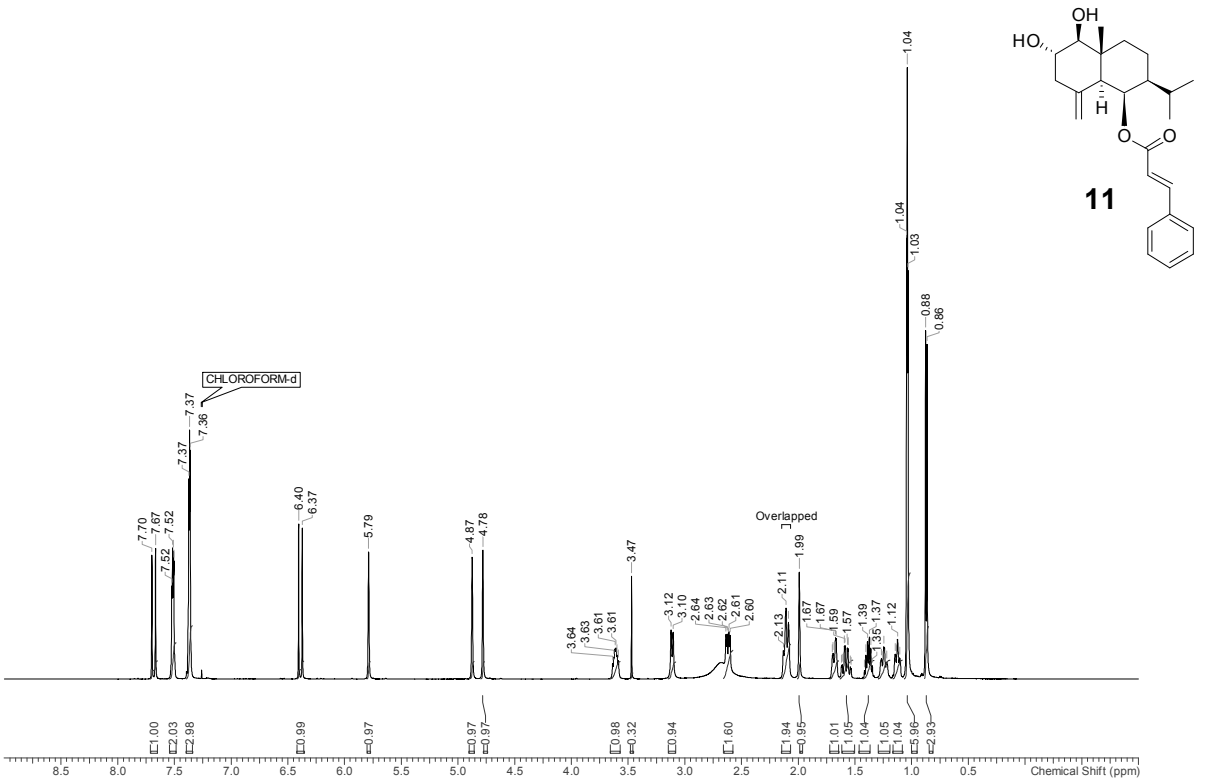


Figure S26. ^{1}H NMR spectrum of compound **11** (500 MHz, $CDCl_3$)

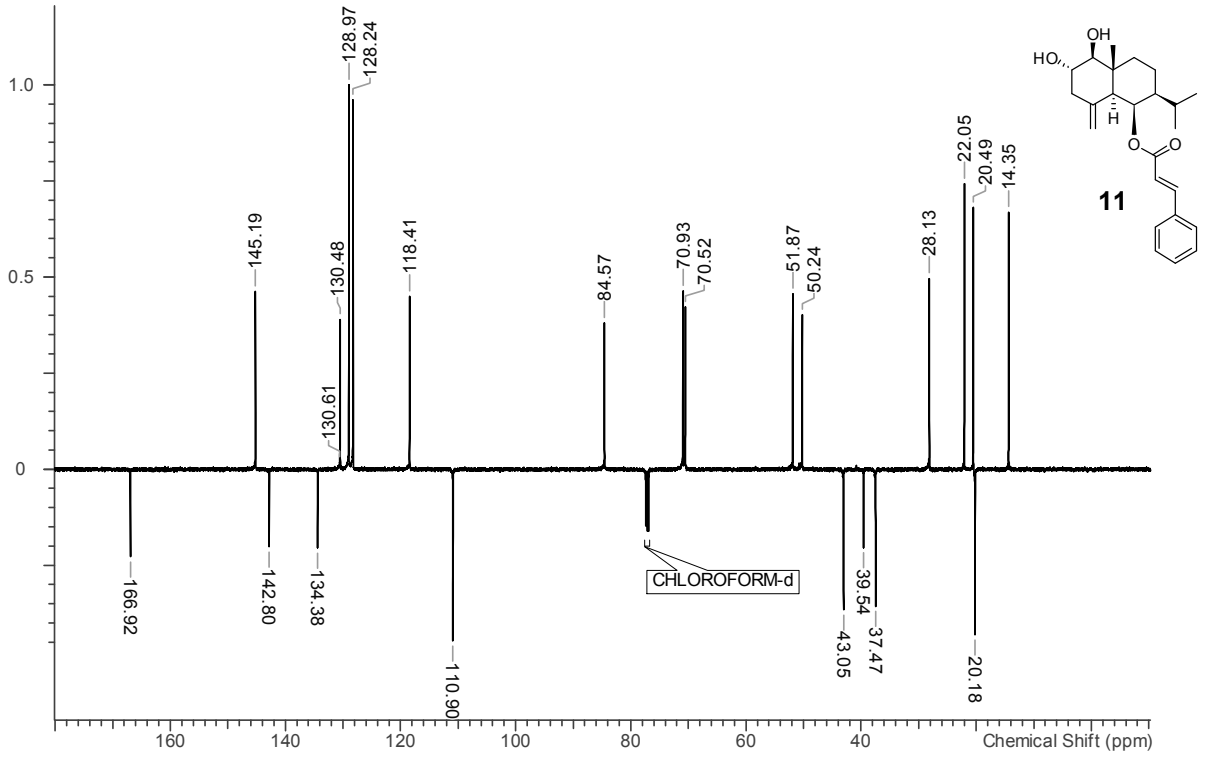


Figure S27. ^{13}C-DEPTq spectrum of compound **11** (125 MHz, $CDCl_3$)

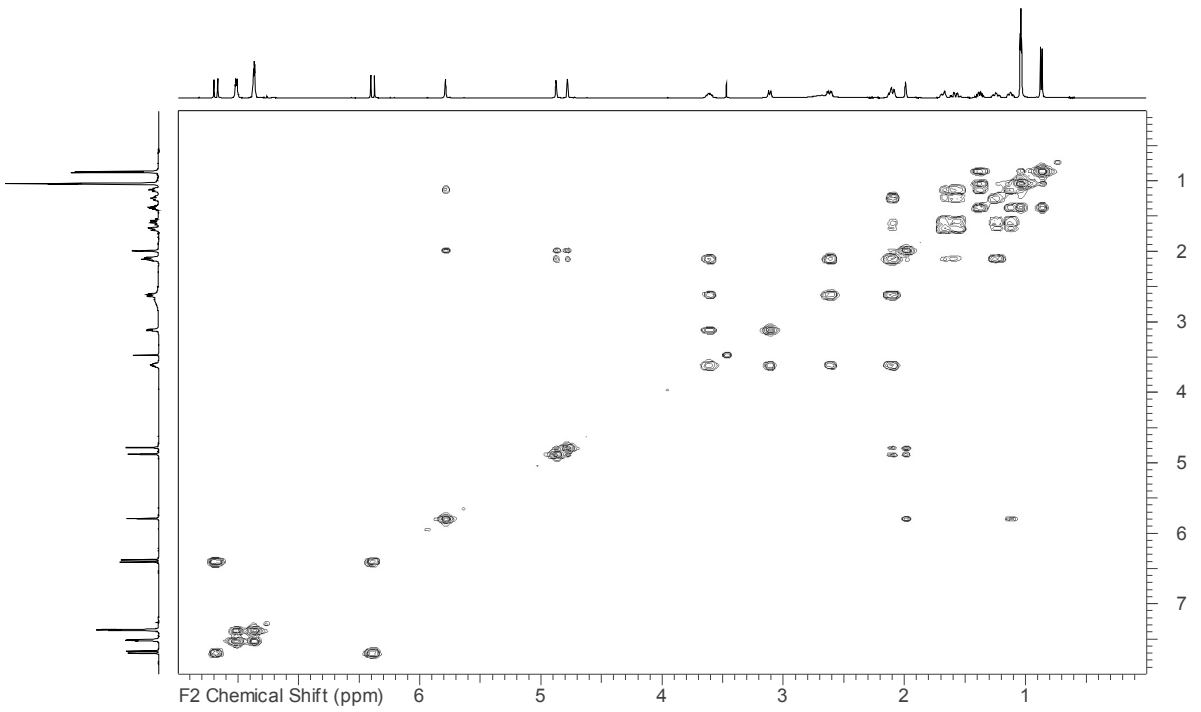


Figure S28. ^{1}H-^{1}H COSY spectrum of compound **11** (500 MHz, $CDCl_3$)

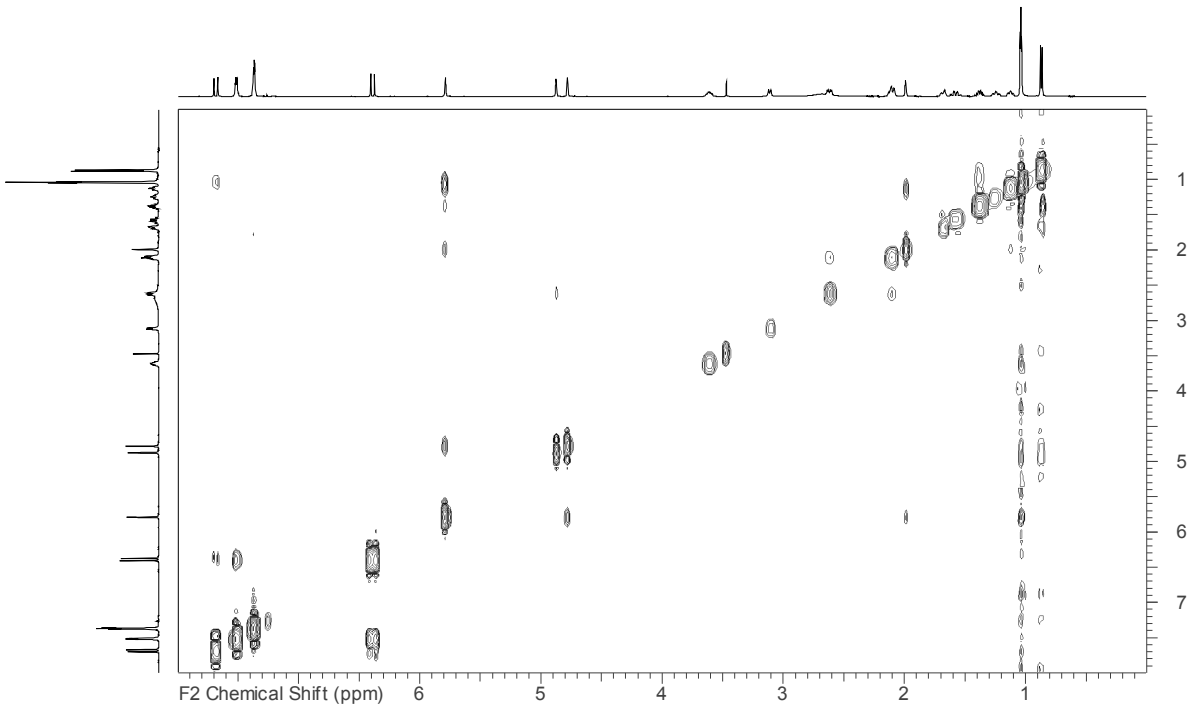


Figure S29. ^{1}H-^{1}H NOESY spectrum of compound **11** (500 MHz, $CDCl_3$)

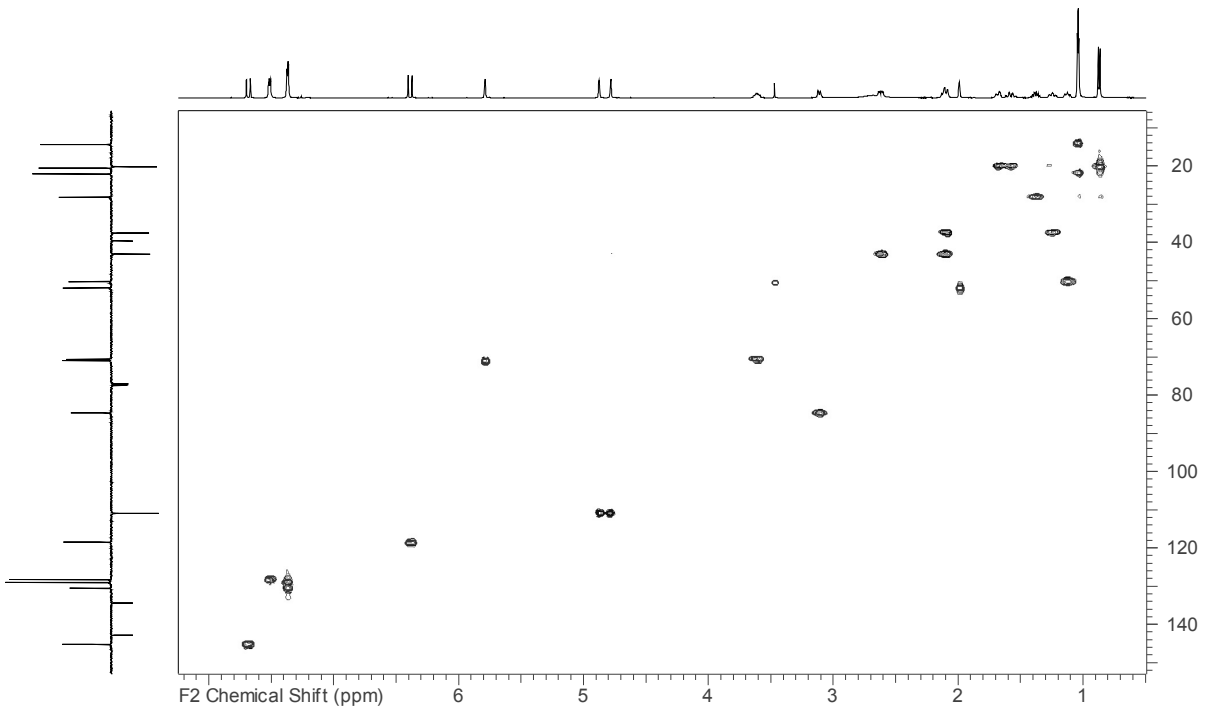


Figure S30. HSQC-DEPT spectrum of compound **11** (500 MHz, $CDCl_3$)

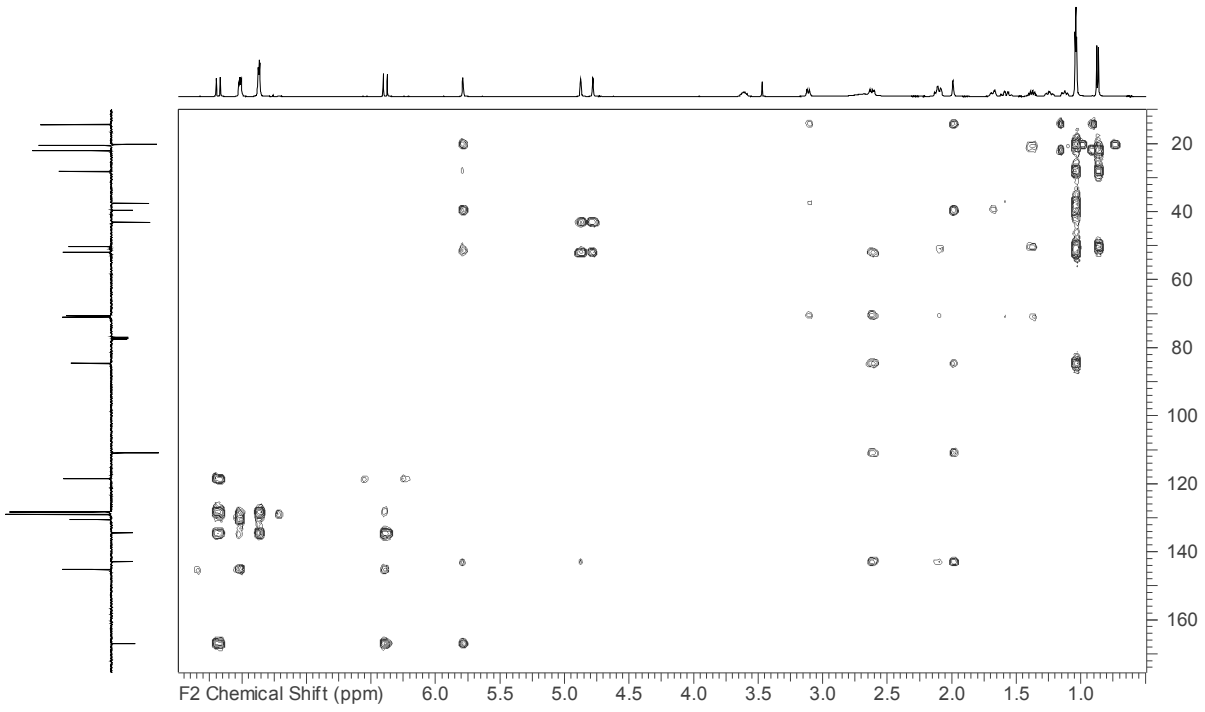


Figure S31. HMBC spectrum of compound **11** (500 MHz, $CDCl_3$)

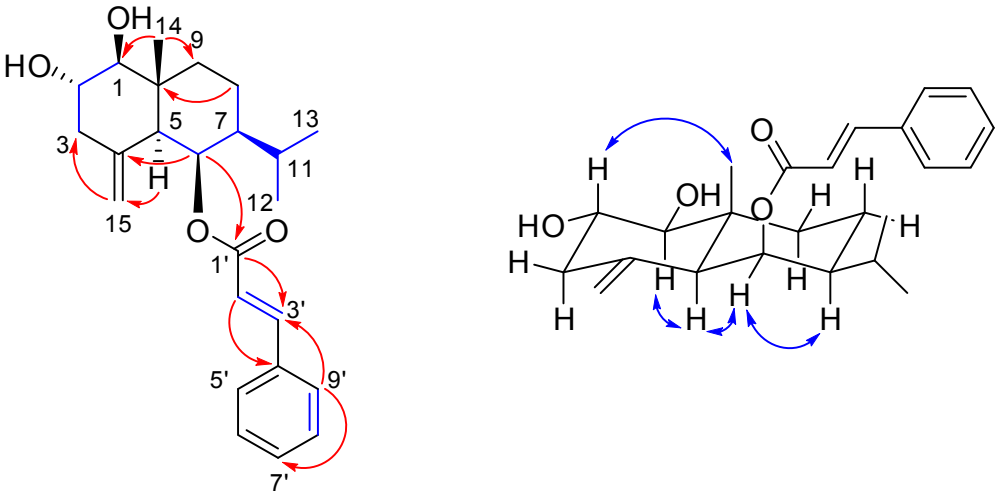


Figure S32. Key COSY (blue bonds), HMBC (red arrows), and NOESY correlations (blue arrows) of compound **11**

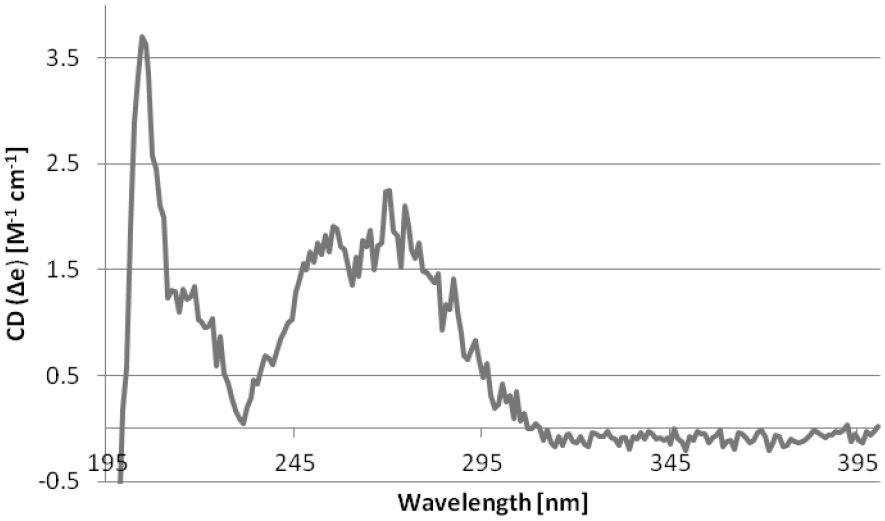


Figure S33. ECD spectrum of compound **11** in MeOH (0.2 mg/mL)

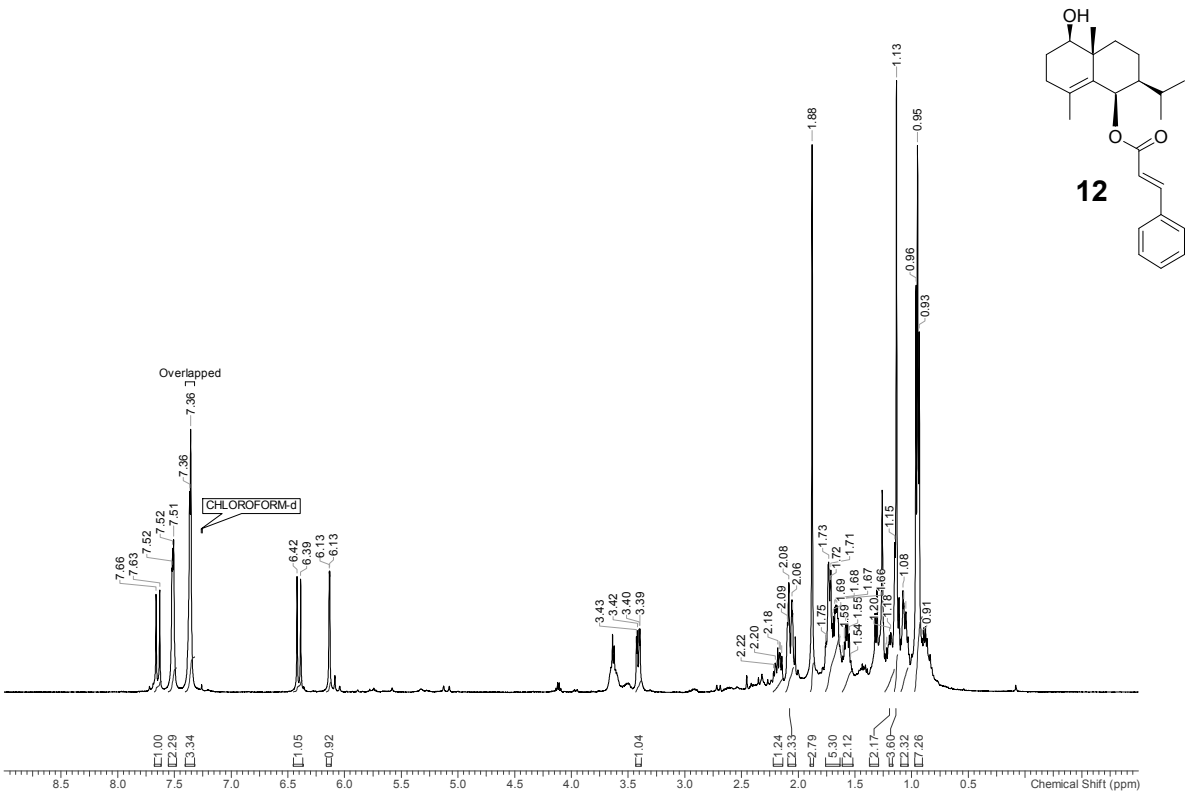


Figure S34. ^{1}H NMR spectrum of compound **12** (500 MHz, $CDCl_3$)

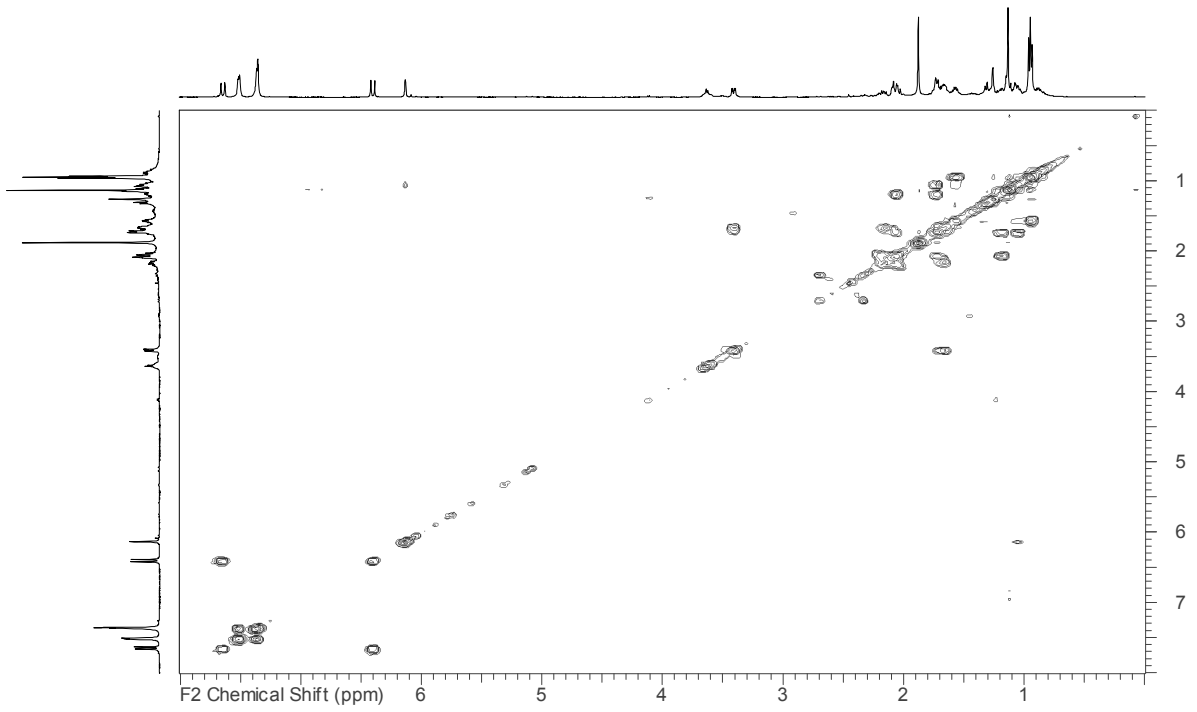


Figure S35. ^{1}H-^{1}H COSY spectrum of compound **12** (500 MHz, $CDCl_3$)

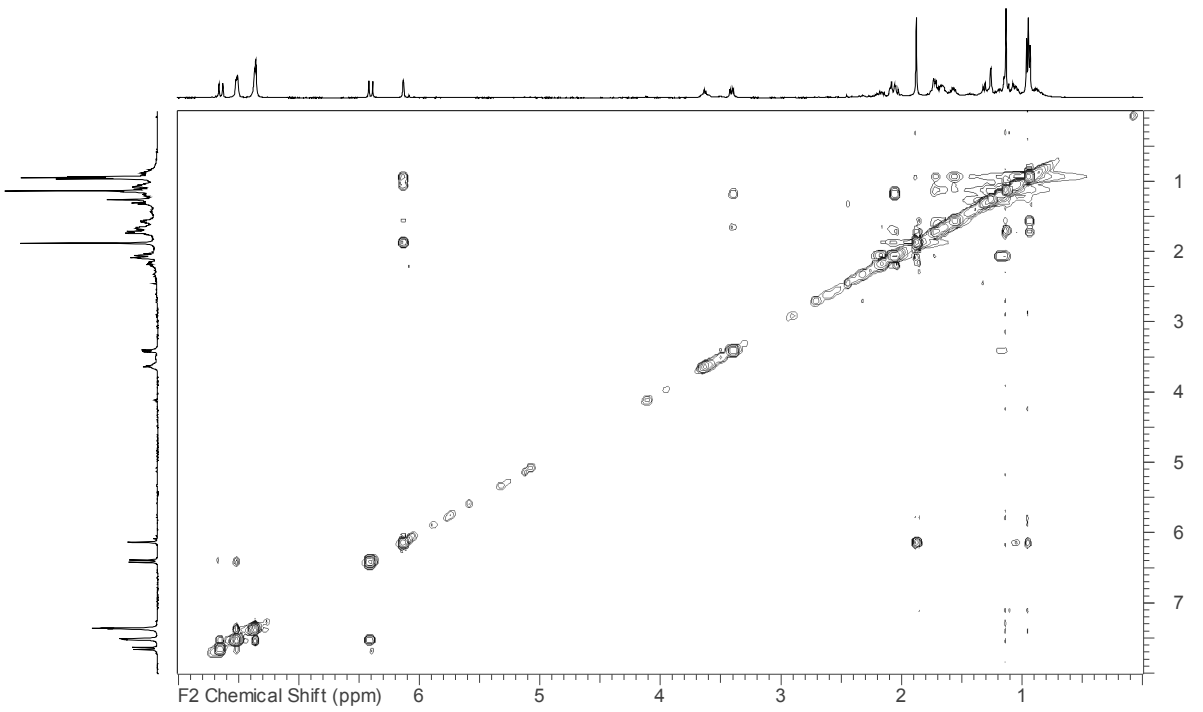


Figure S36. ^{1}H-^{1}H NOESY spectrum of compound **12** (500 MHz, $CDCl_3$)

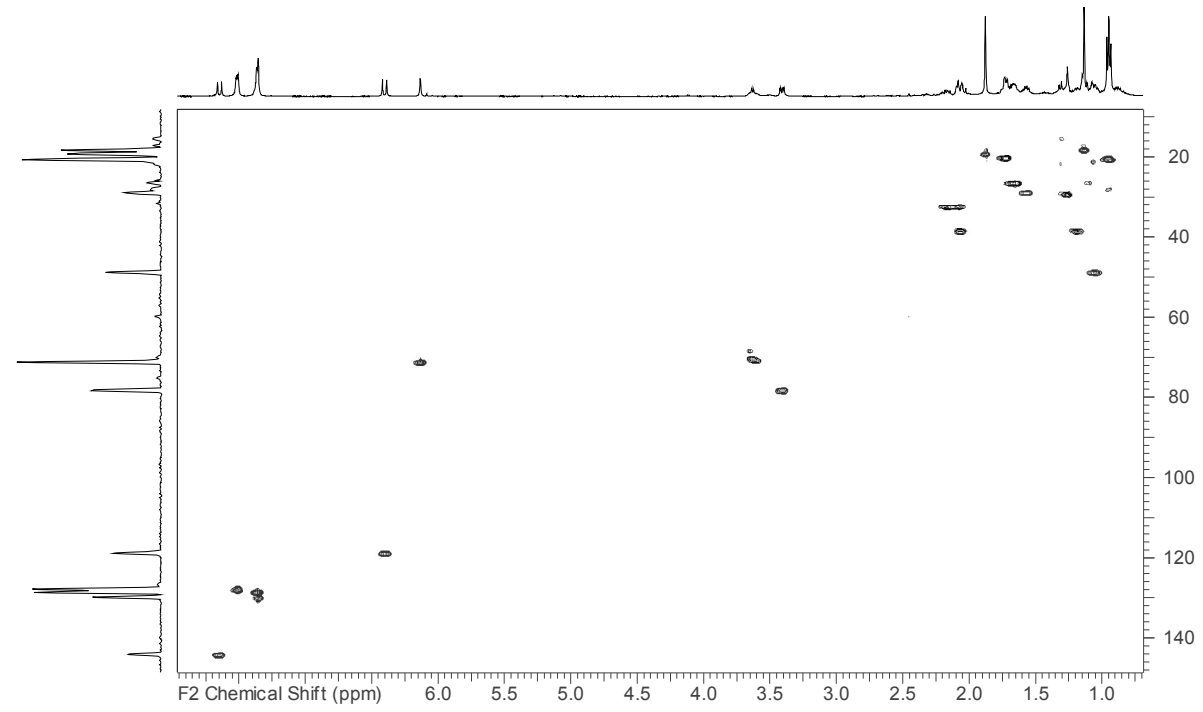


Figure S37. HSQC spectrum of compound **12** (500 MHz, $CDCl_3$)

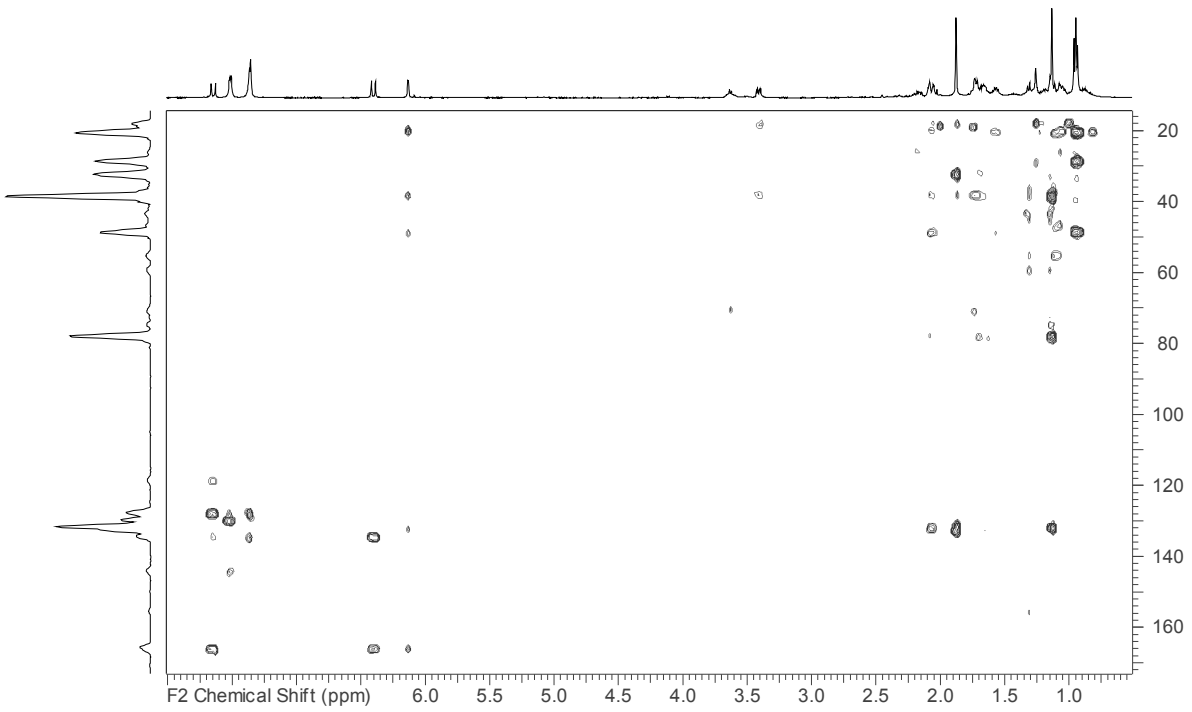


Figure S38. HMBC spectrum of compound **12** (500 MHz, $CDCl_3$)

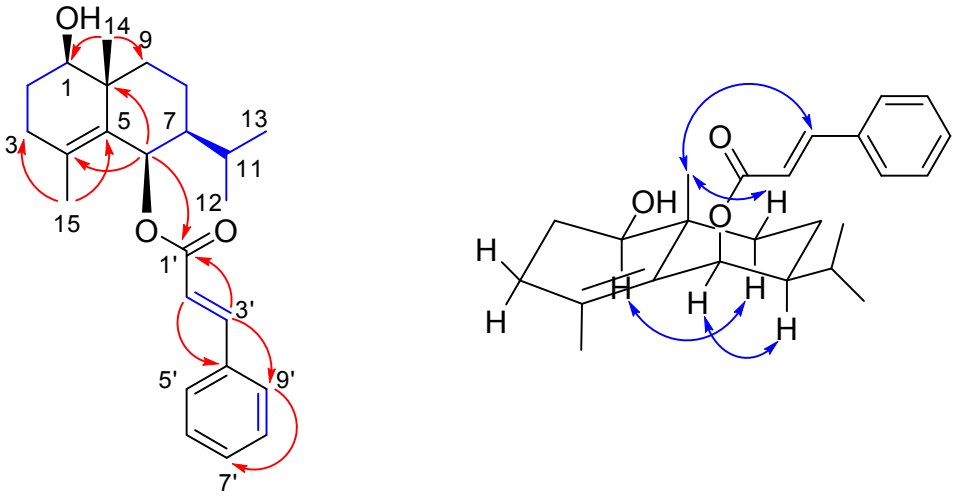


Figure S39. Key COSY (blue bonds), HMBC (red arrows), and NOESY correlations (blue arrows) of compound **12**

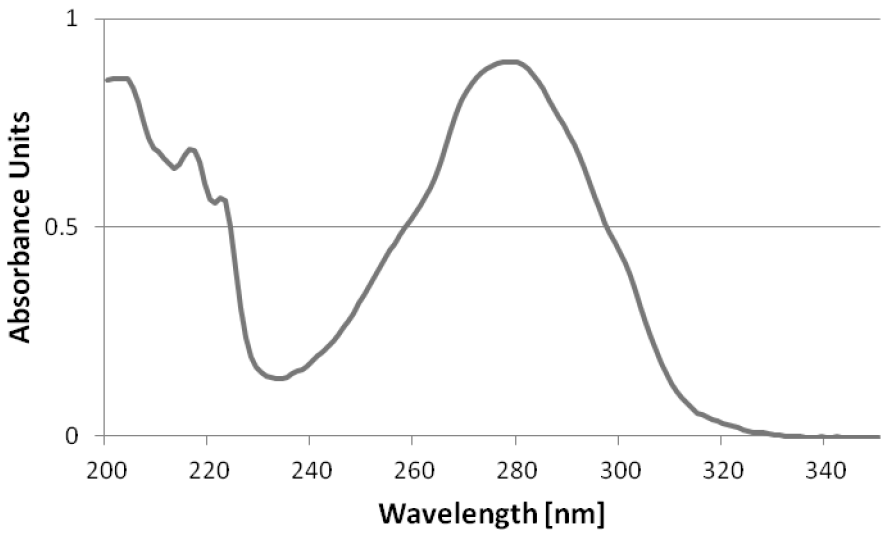


Figure S40. UV spectrum of compound **12** in MeOH (0.2 mg/mL)

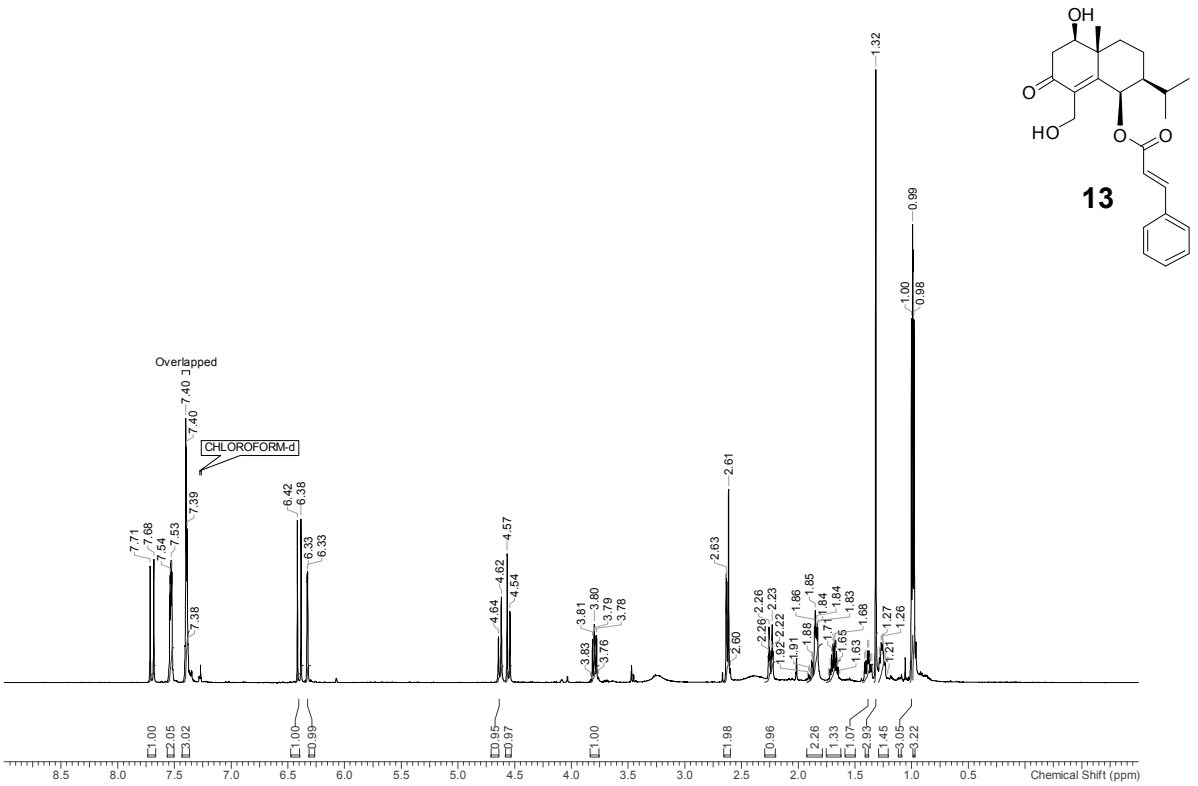


Figure S41. ^{1}H NMR spectrum of compound **13** (500 MHz, $CDCl_3$)

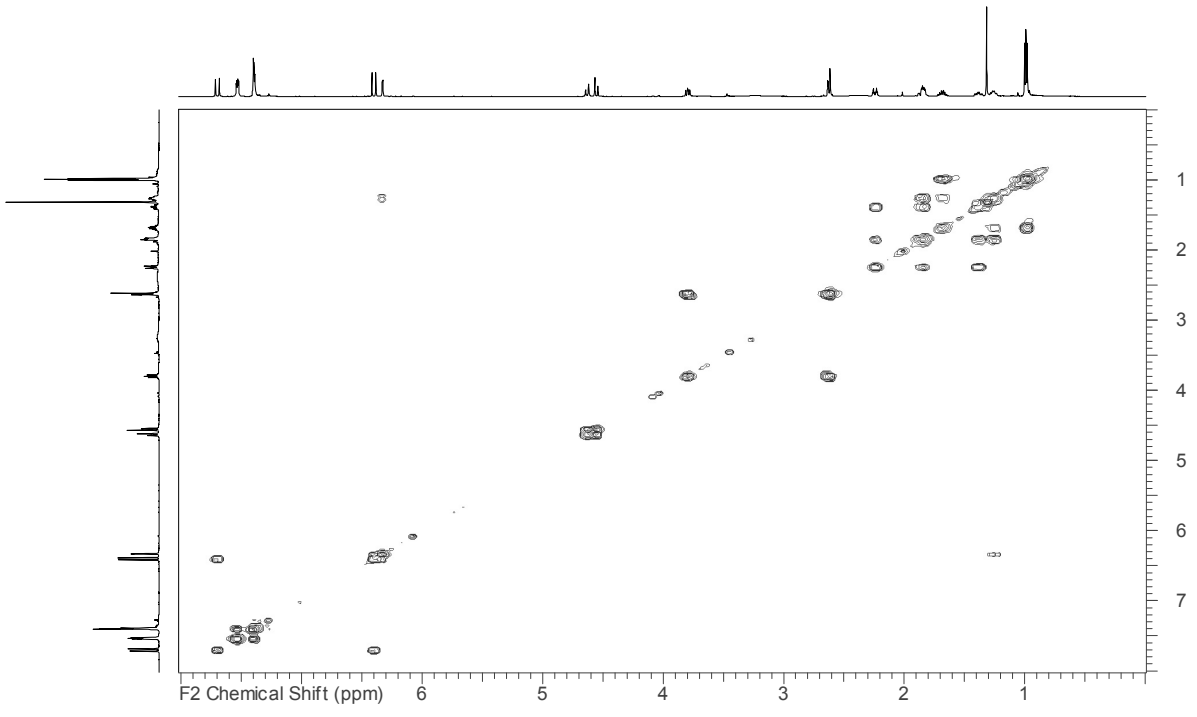


Figure S42. ^{1}H-^{1}H COSY spectrum of compound **13** (500 MHz, $CDCl_3$)

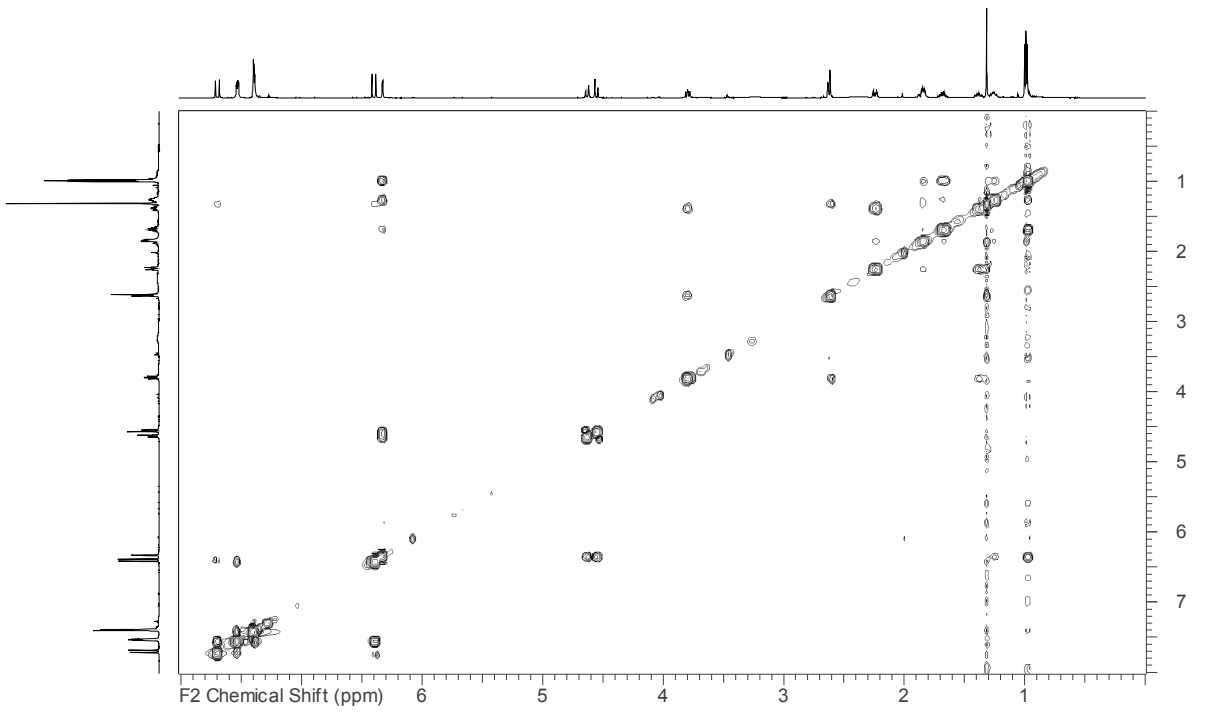


Figure S43. ^{1}H-^{1}H NOESY spectrum of compound **13** (500 MHz, $CDCl_3$)

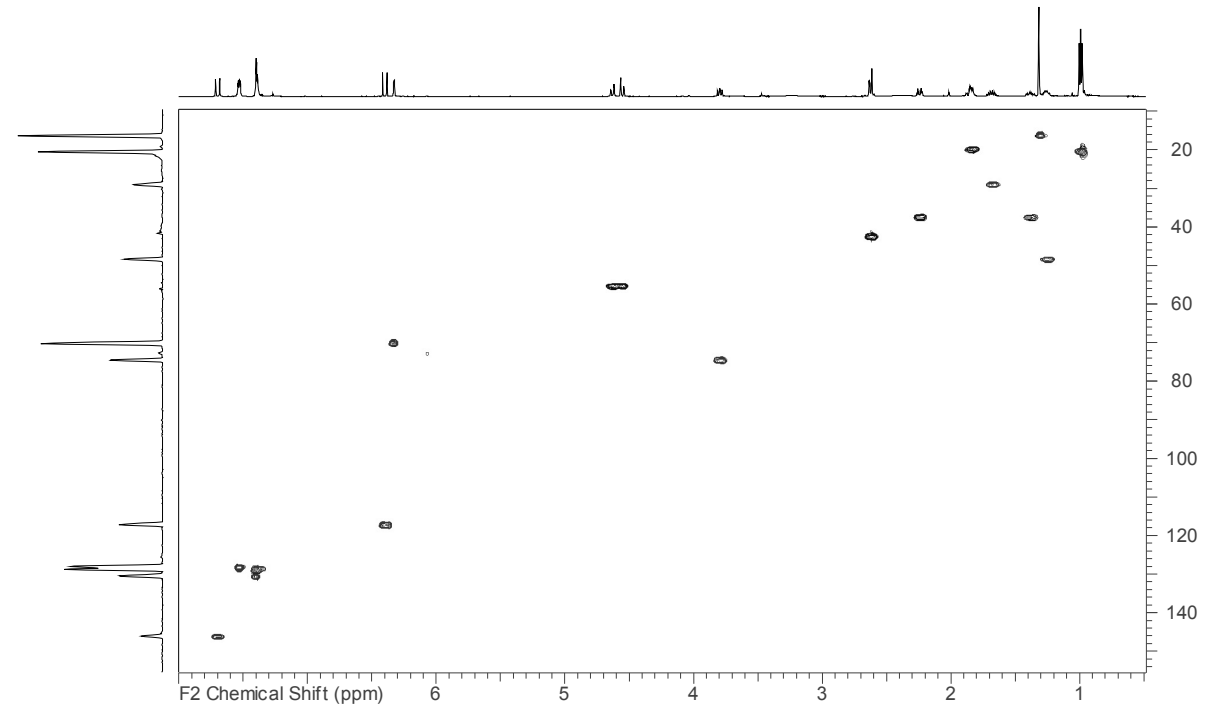


Figure S44. HSQC spectrum of compound **13** (500 MHz, $CDCl_3$)

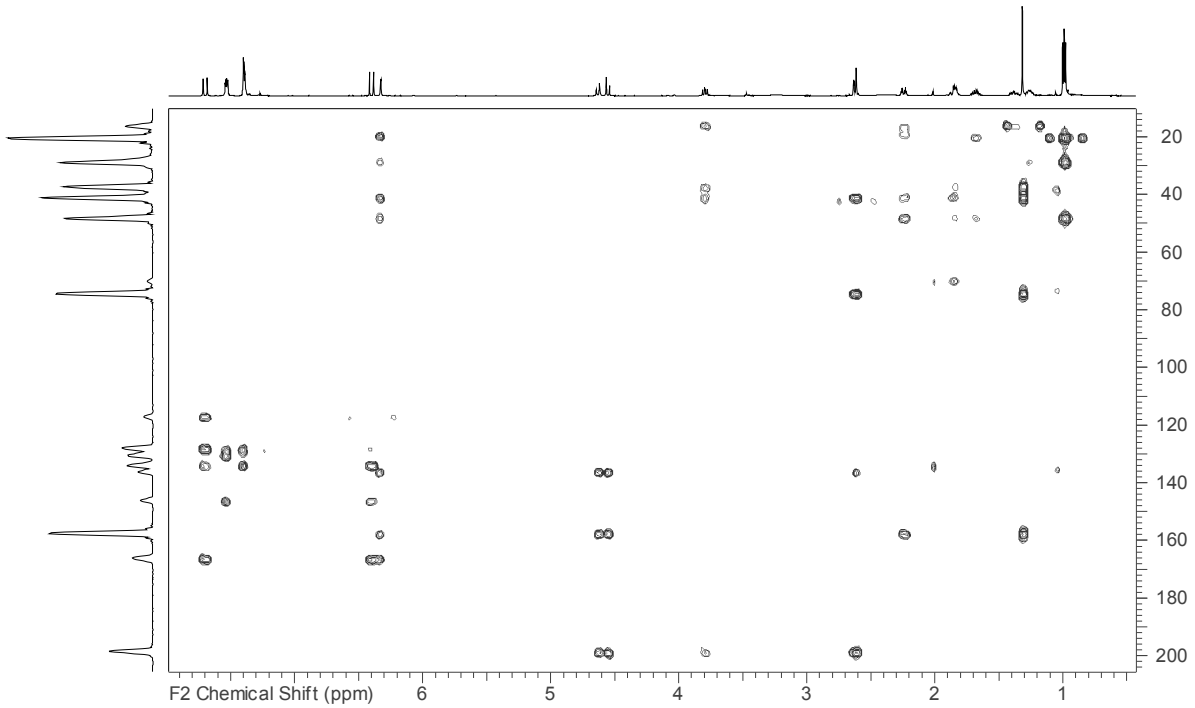


Figure S45. HMBC spectrum of compound **13** (500 MHz, $CDCl_3$)

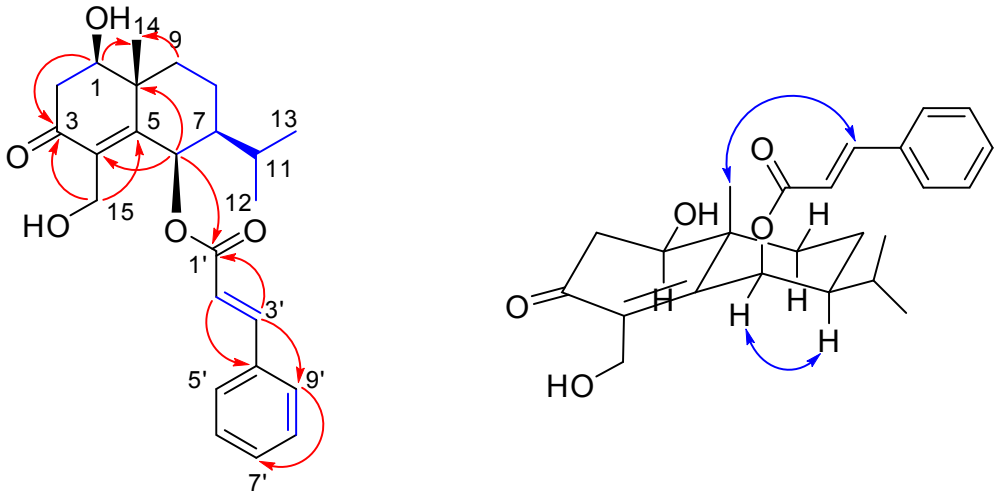


Figure S46. Key COSY (blue bonds), HMBC (red arrows), and NOESY correlations (blue arrows) of compound **13**

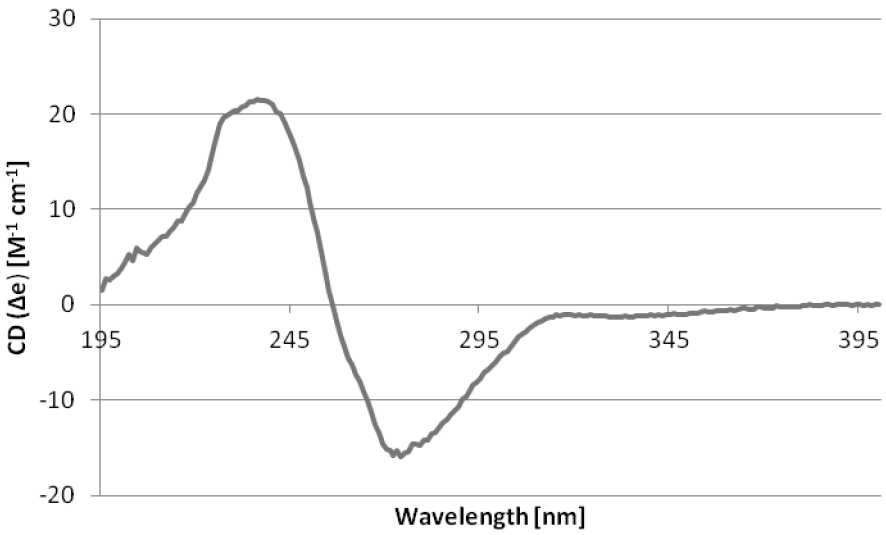


Figure S47. ECD spectrum of compound **13** in MeOH (0.2 mg/mL)

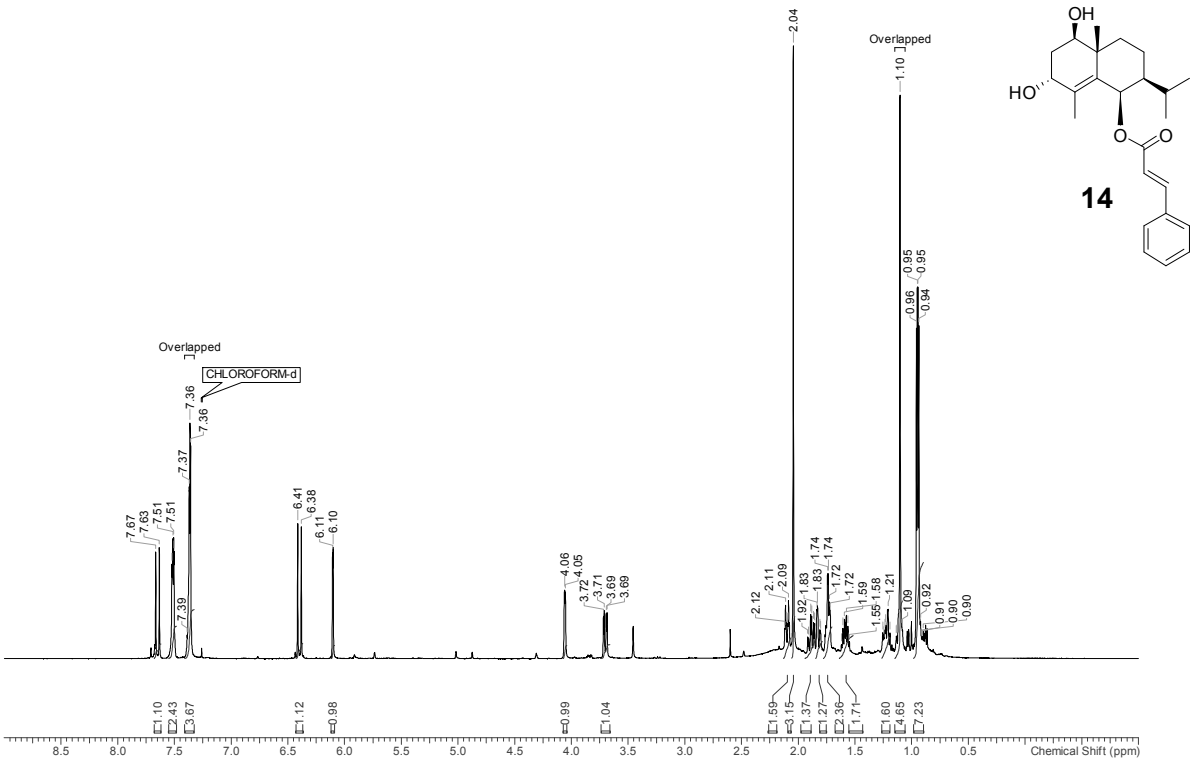


Figure S48. ^{1}H NMR spectrum of compound **14** (500 MHz, $CDCl_3$)

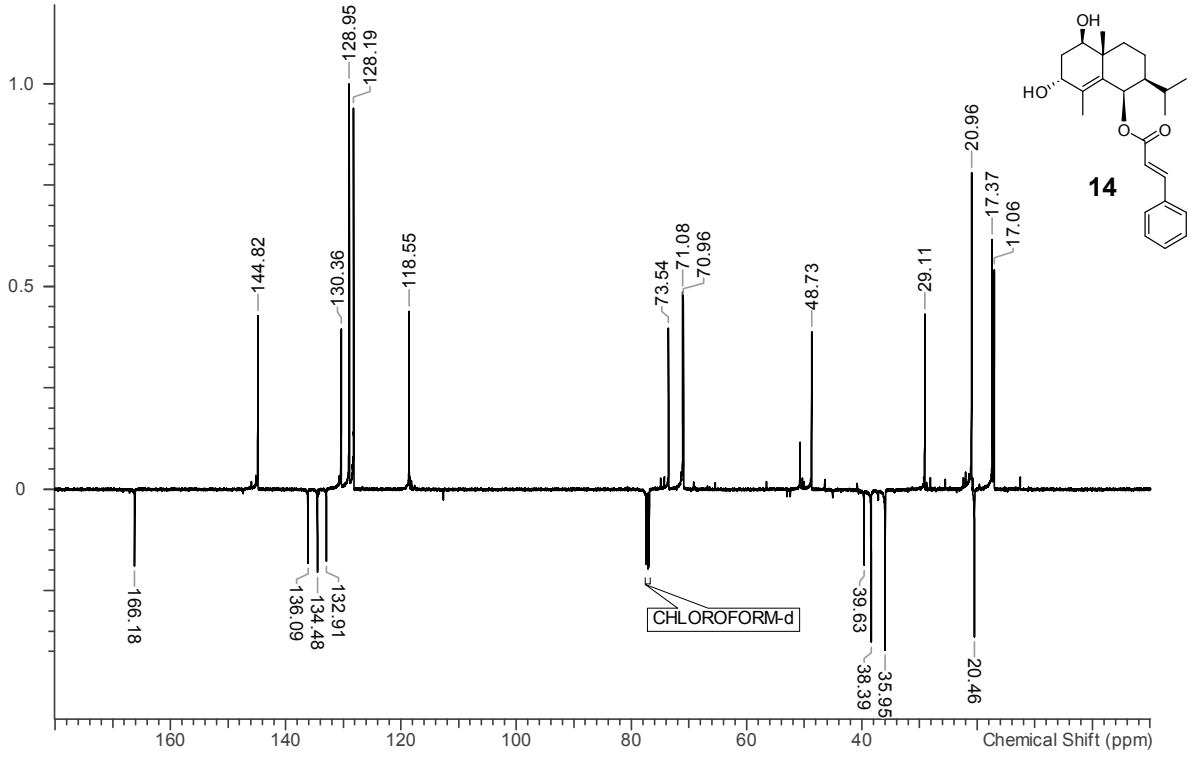


Figure S49. ^{13}C-DEPTq spectrum of compound **14** (125 MHz, $CDCl_3$)

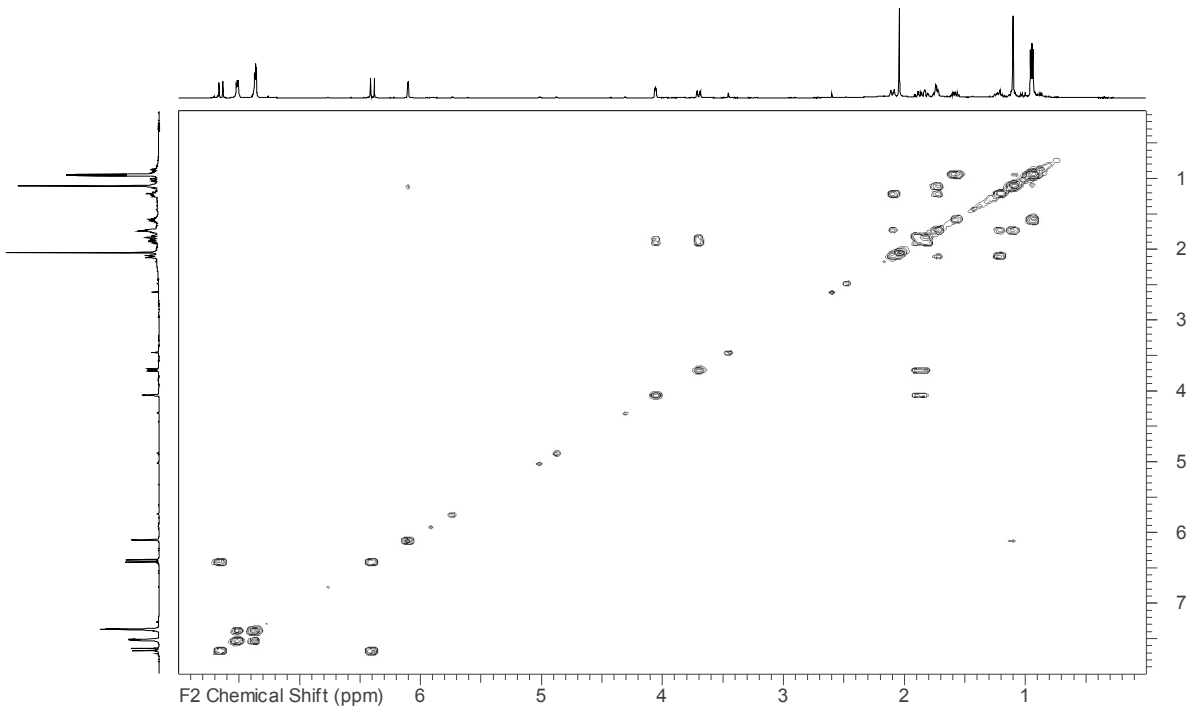


Figure S50. ^{1}H-^{1}H COSY spectrum of compound **14** (500 MHz, $CDCl_3$)

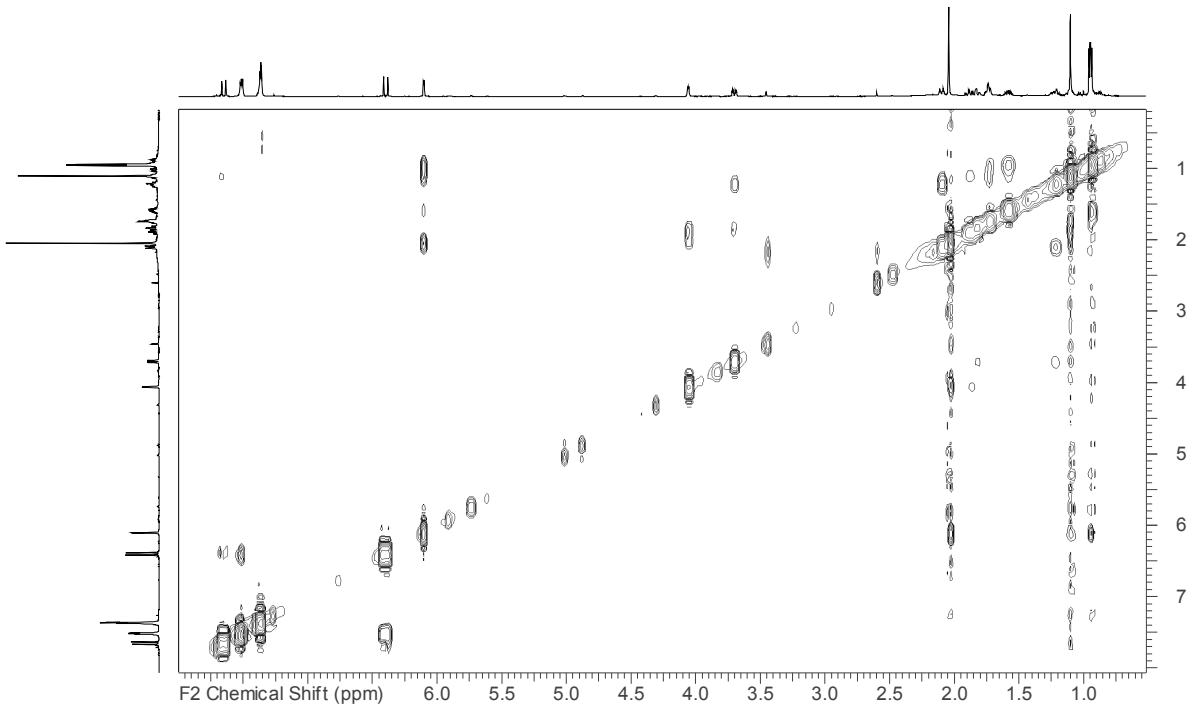


Figure S51. ^{1}H-^{1}H NOESY spectrum of compound **14** (500 MHz, $CDCl_3$)

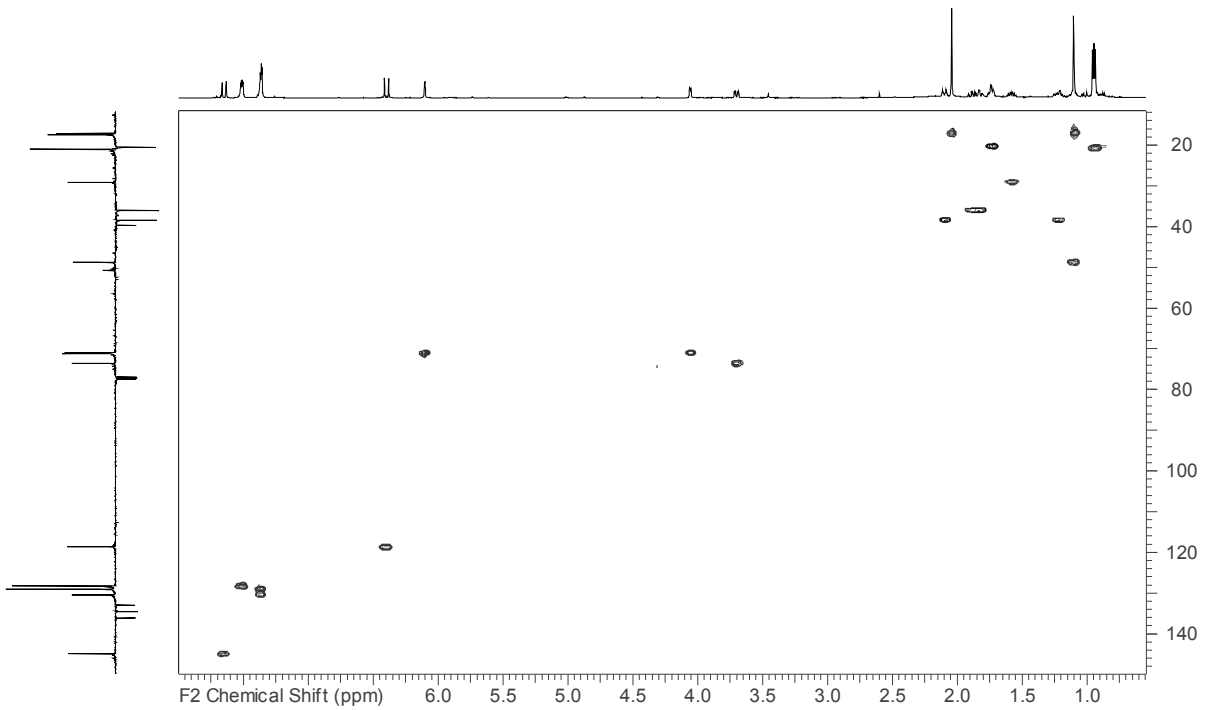


Figure S52. HSQC-DEPT spectrum of compound **14** (500 MHz, $CDCl_3$)

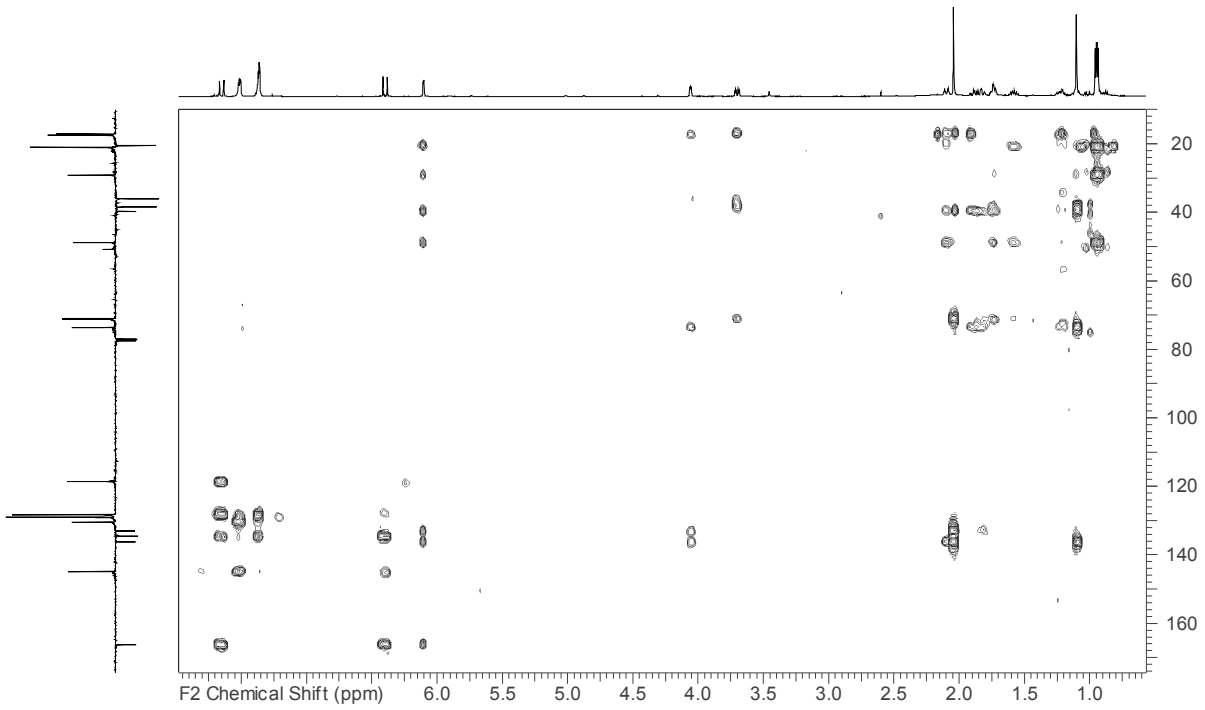


Figure S53. HMBC spectrum of compound **14** (500 MHz, $CDCl_3$)

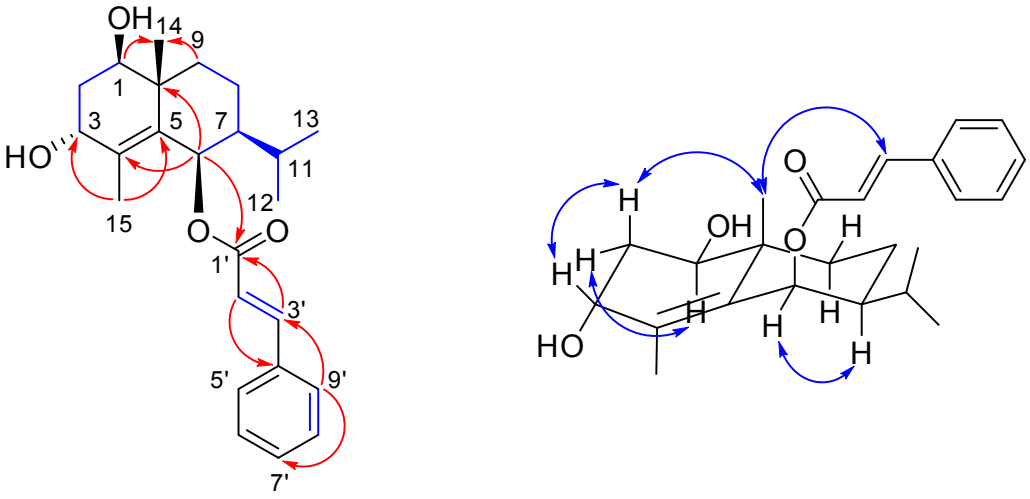


Figure S54. Key COSY (blue bonds), HMBC (red arrows), and NOESY correlations (blue arrows) of compound **14**

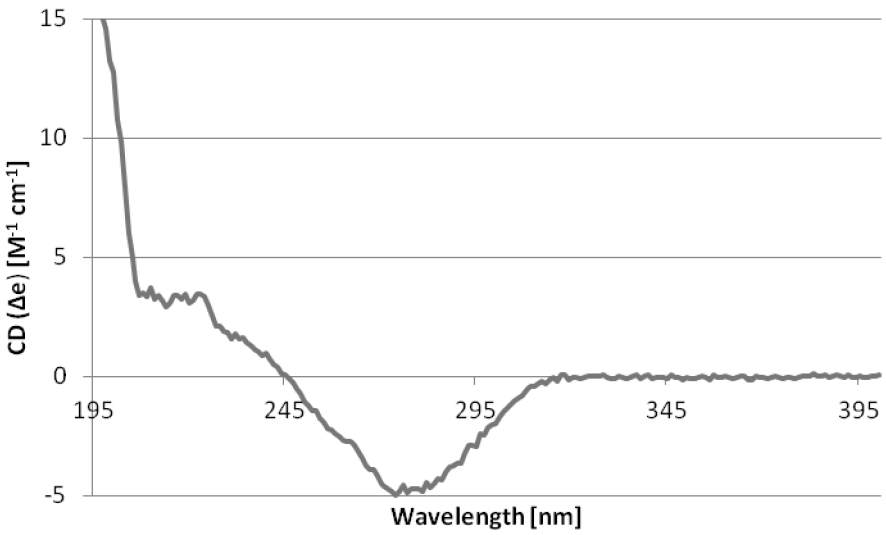


Figure S55. ECD spectrum of compound **14** in MeOH (0.2 mg/mL)

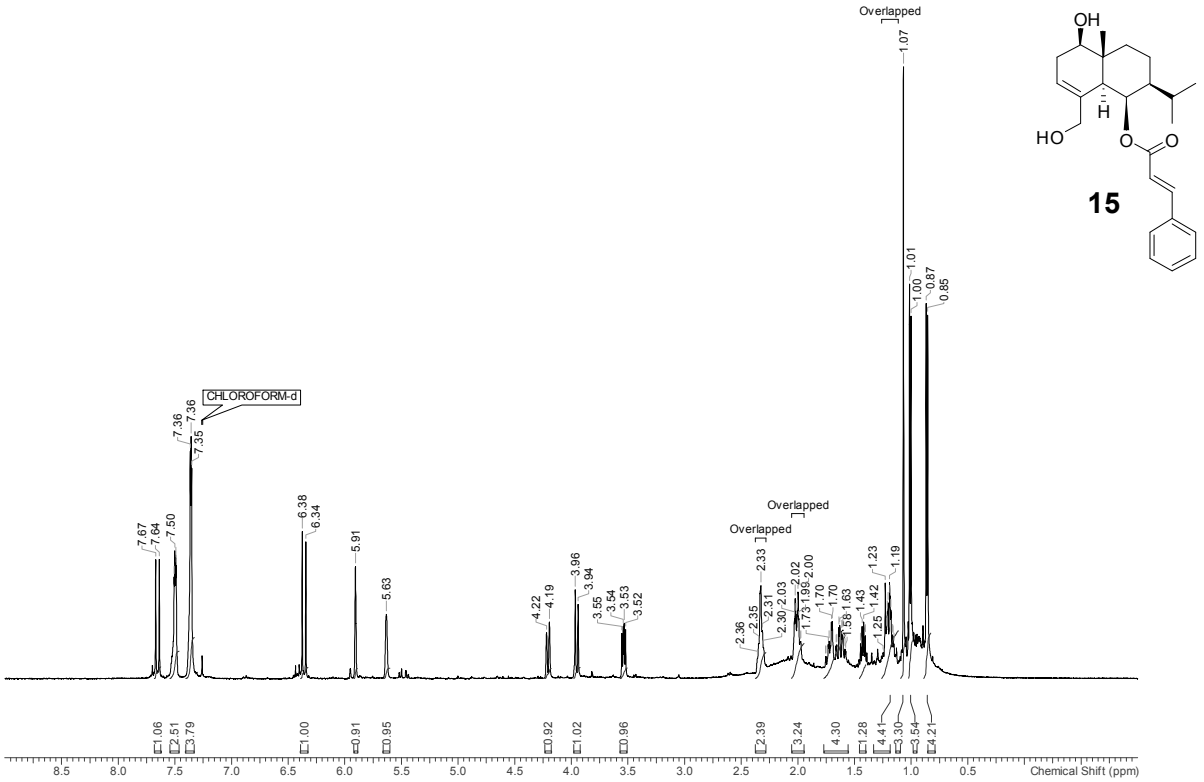


Figure S56. ^{1}H NMR spectrum of compound **15** (500 MHz, $CDCl_3$)

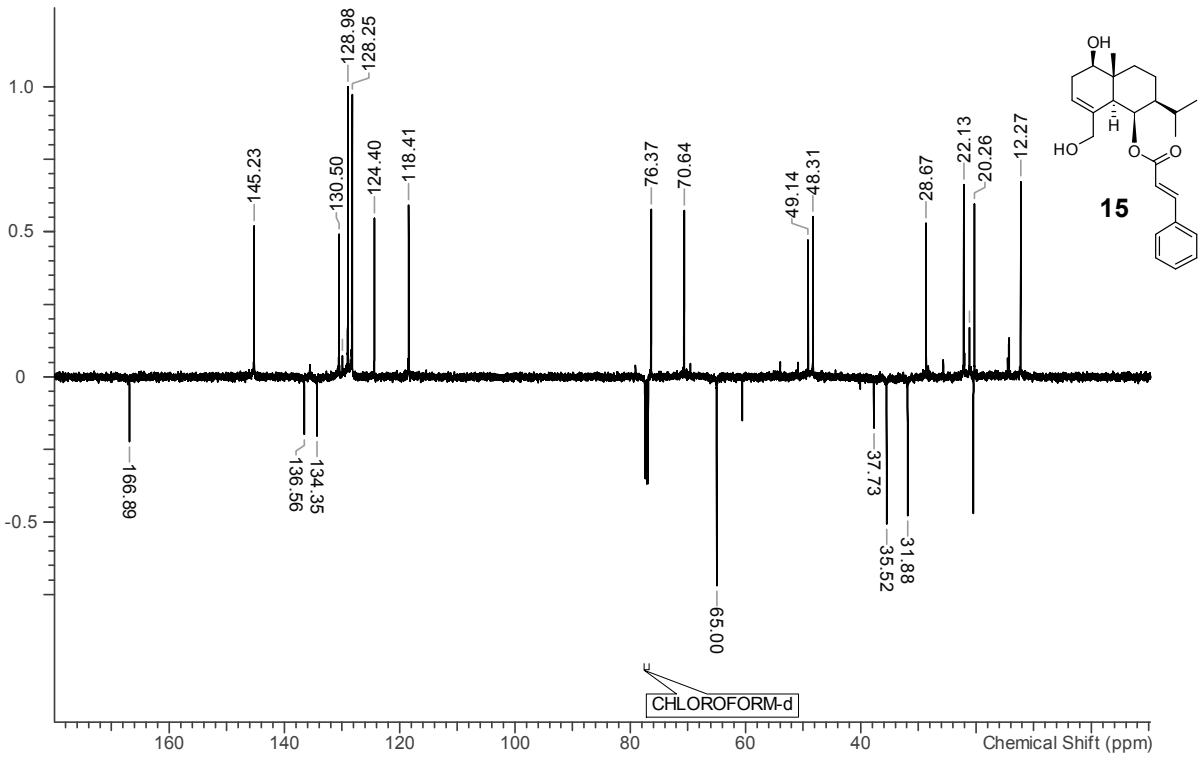


Figure S57. ^{13}C-DEPTq spectrum of compound **15** (125 MHz, $CDCl_3$)

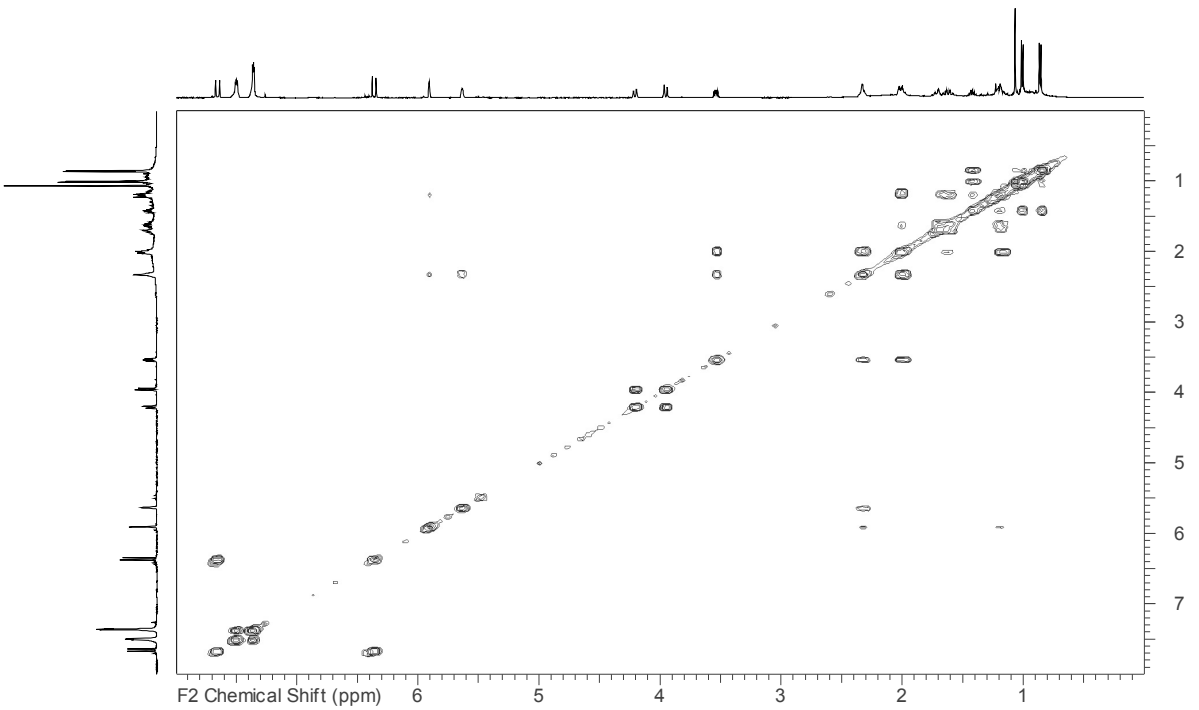


Figure S58. ^{1}H-^{1}H COSY spectrum of compound **15** (500 MHz, $CDCl_3$)

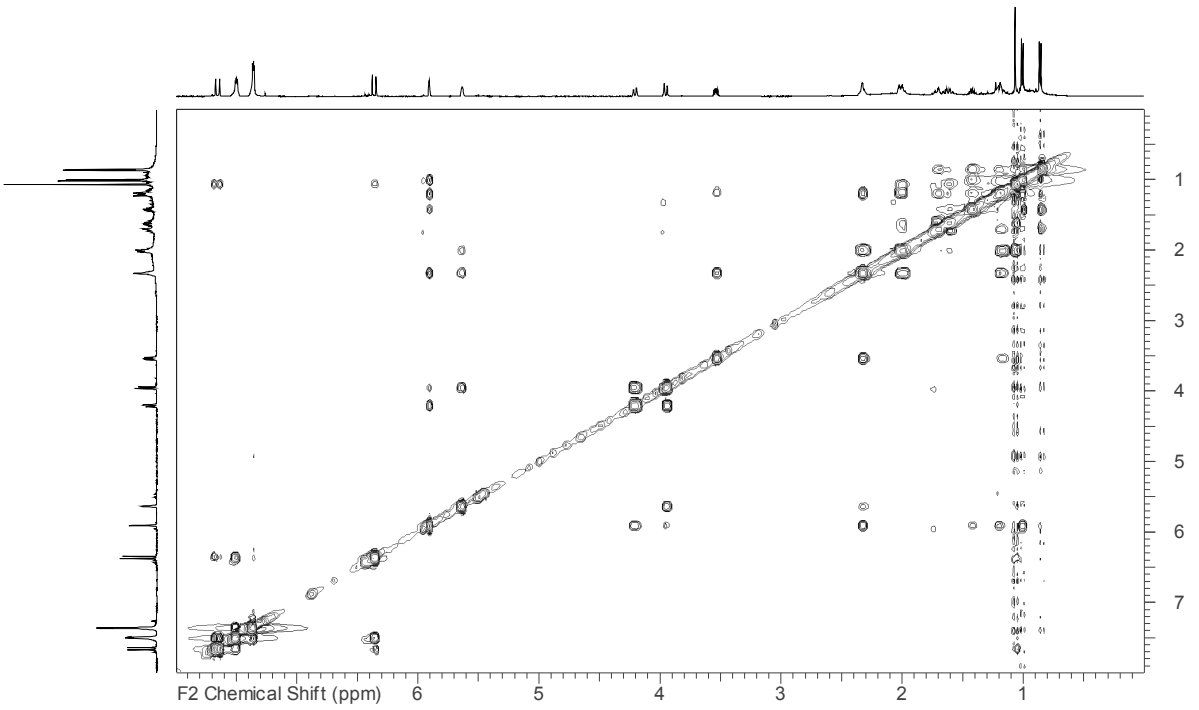


Figure S59. ^{1}H-^{1}H NOESY spectrum of compound **15** (500 MHz, $CDCl_3$)

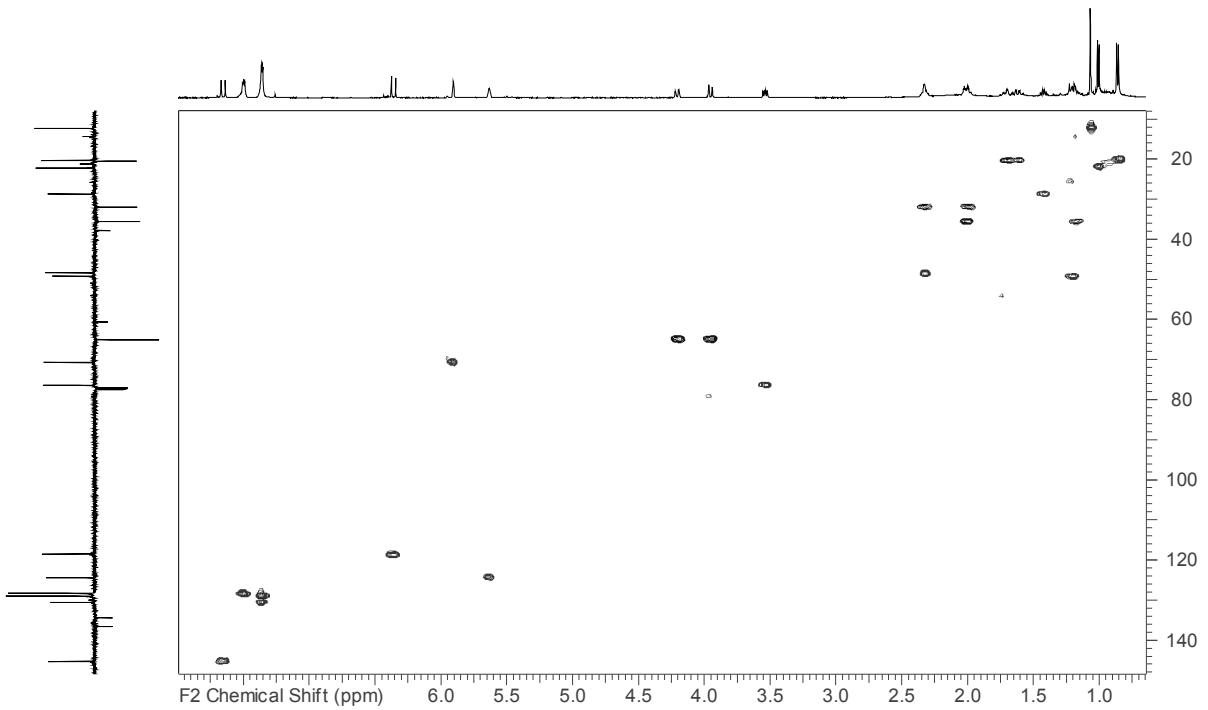


Figure S60. HSQC-DEPT spectrum of compound **15** (500 MHz, $CDCl_3$)

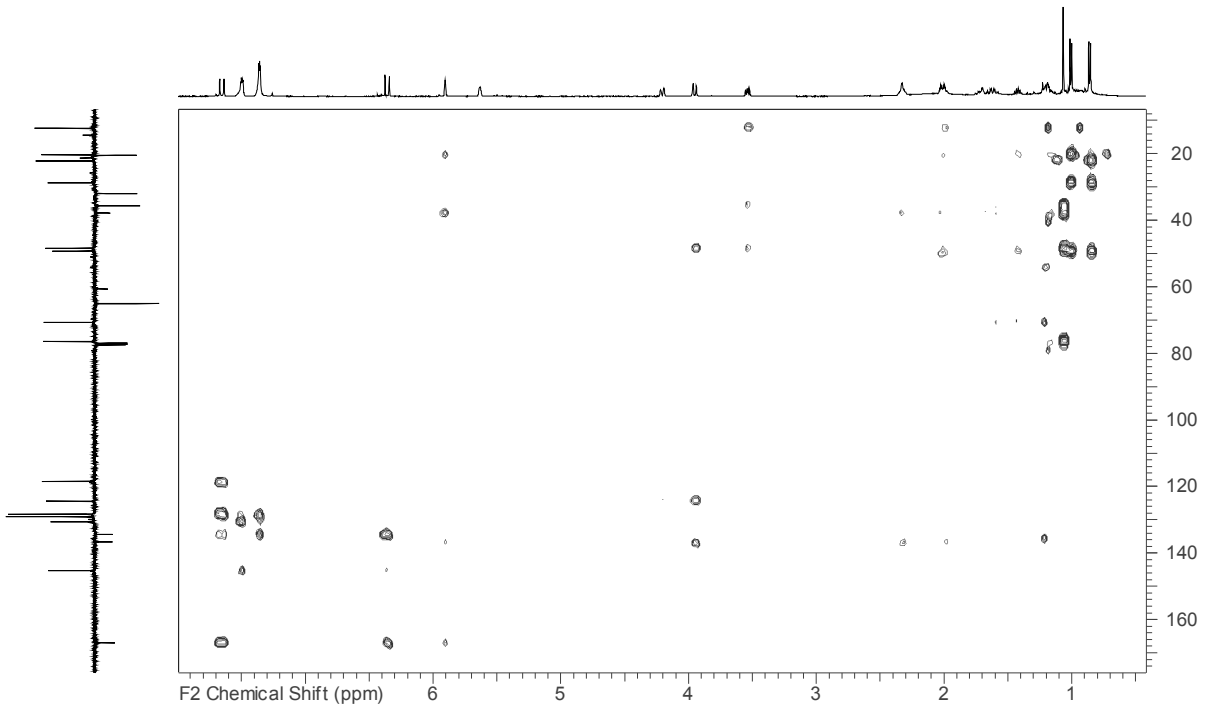


Figure S61. HMBC spectrum of compound **15** (500 MHz, $CDCl_3$)

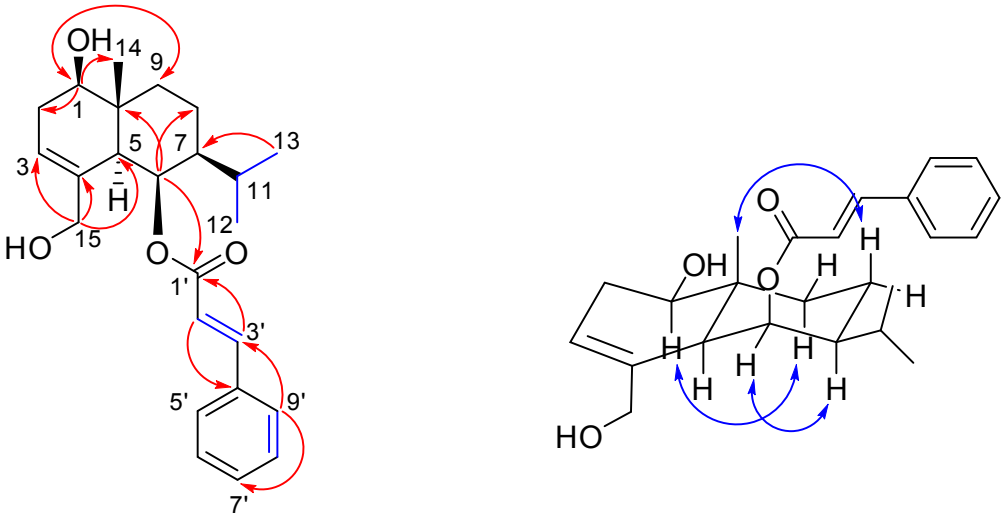


Figure S62. Key COSY (blue bonds), HMBC (red arrows), and NOESY correlations (blue arrows) of compound **15**

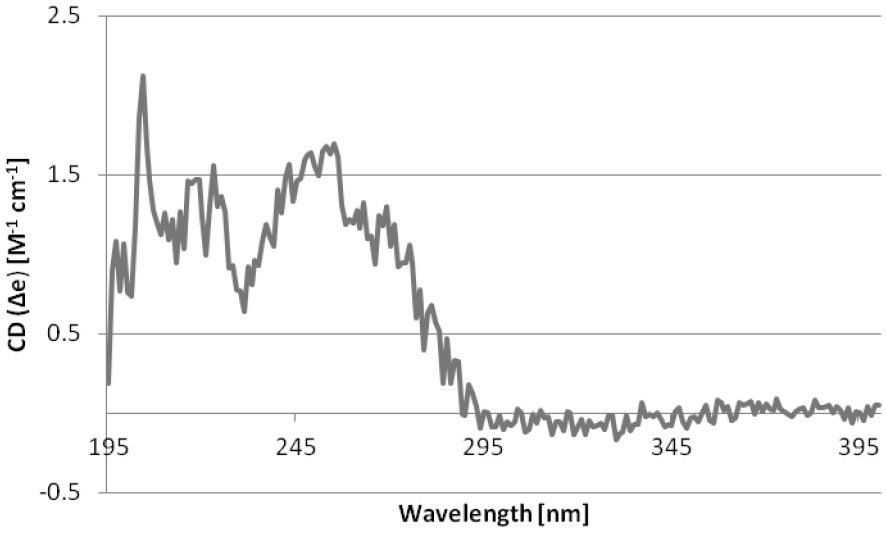


Figure S63. ECD spectrum of compound **15** in MeOH (0.2 mg/mL)

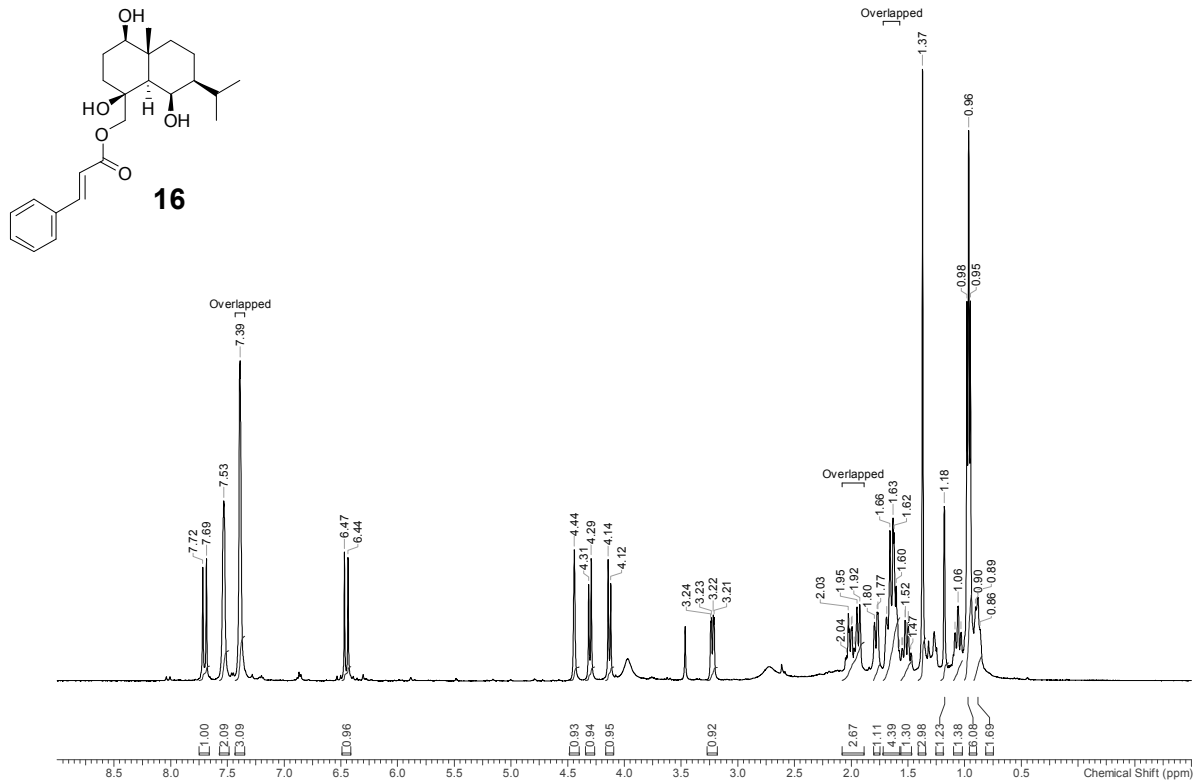


Figure S64. ^{1}H NMR spectrum of compound **16** (500 MHz, $CDCl_3$)

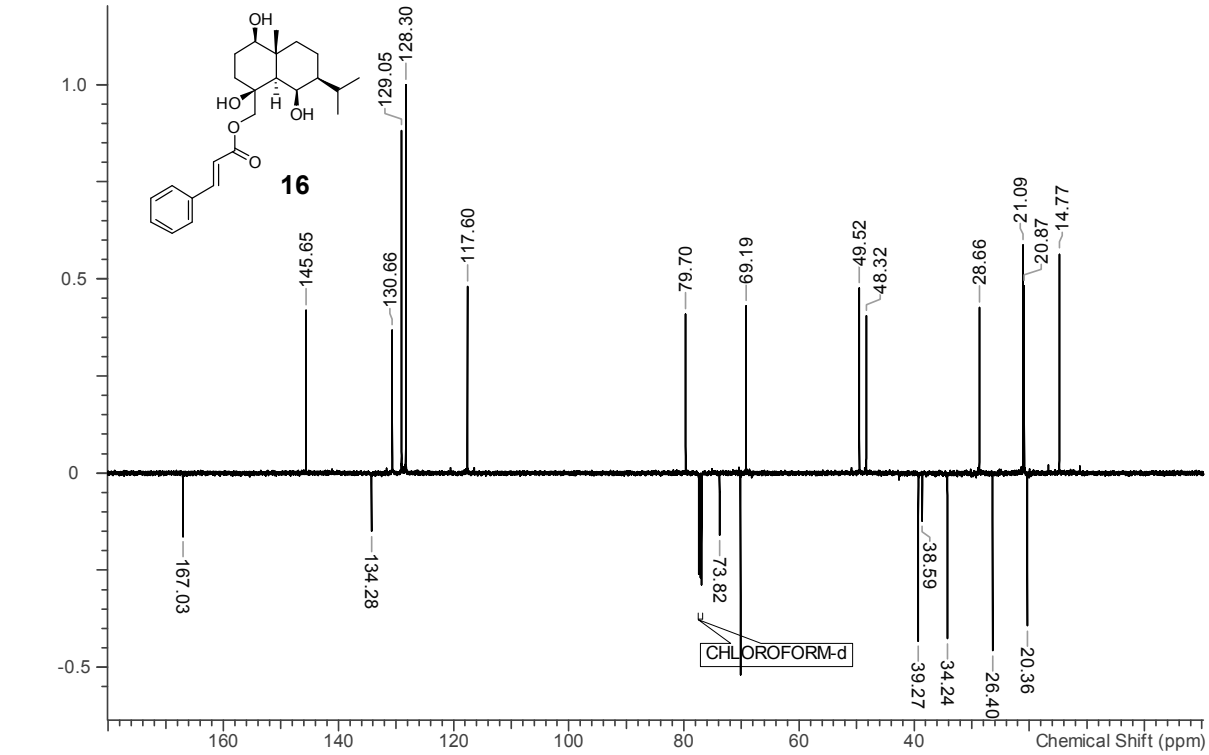


Figure S65. ^{13}C-DEPTq spectrum of compound **16** (125 MHz, $CDCl_3$)

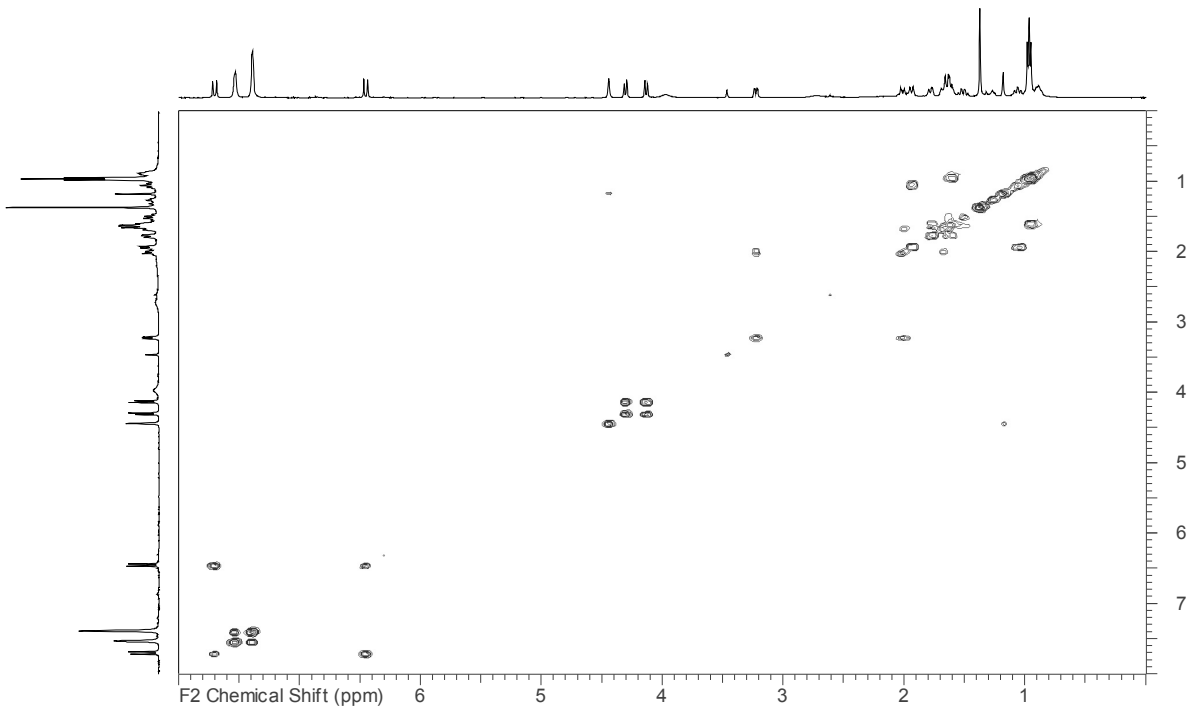


Figure S66. ^{1}H-^{1}H COSY spectrum of compound **16** (500 MHz, $CDCl_3$)

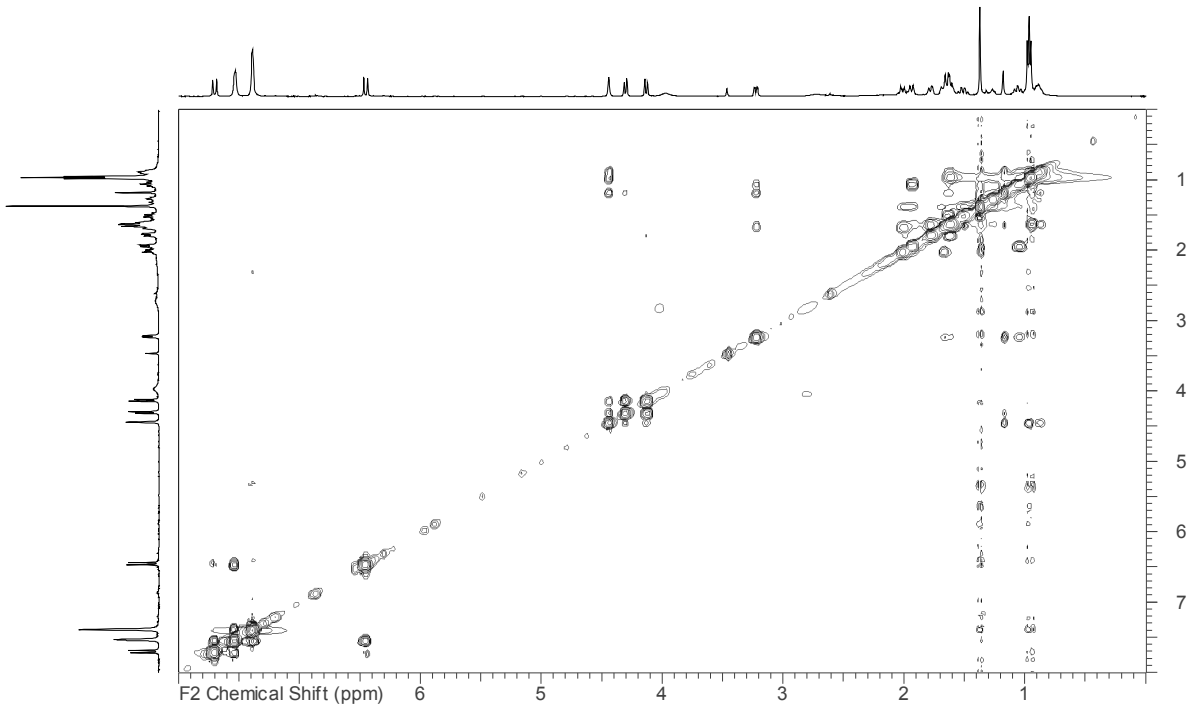


Figure S67. ^{1}H-^{1}H NOESY spectrum of compound **16** (500 MHz, $CDCl_3$)

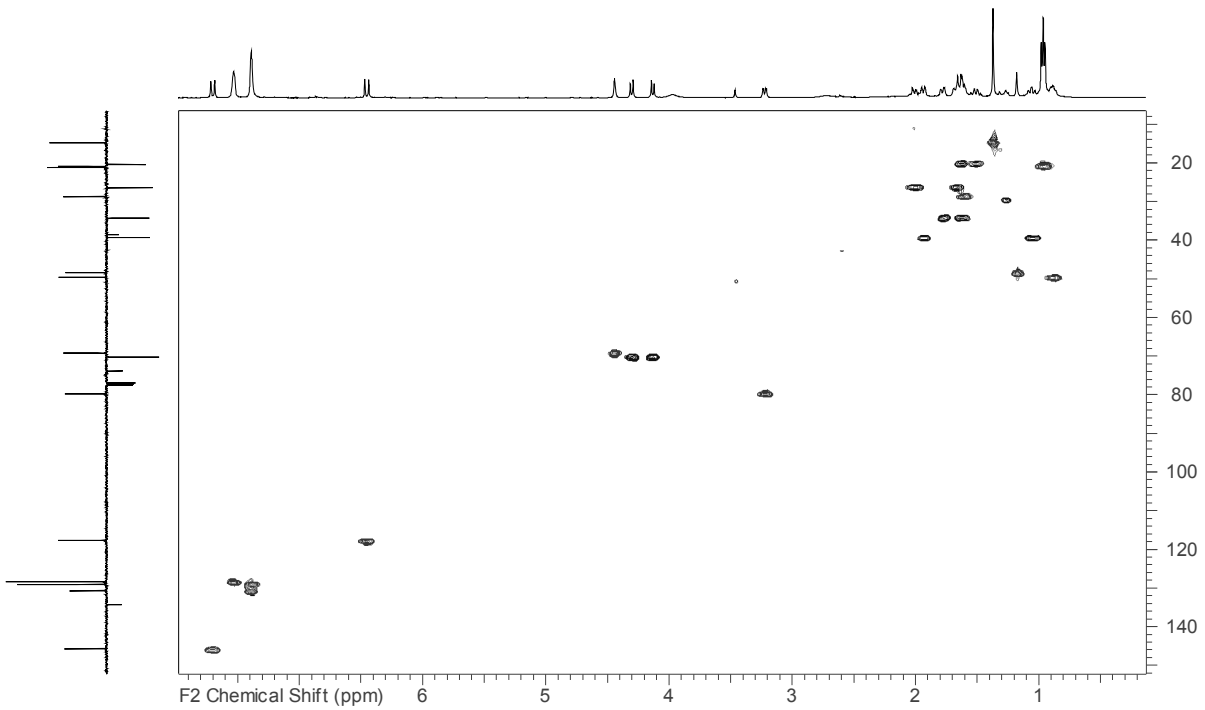


Figure S68. HSQC-DEPT spectrum of compound **16** (500 MHz, $CDCl_3$)

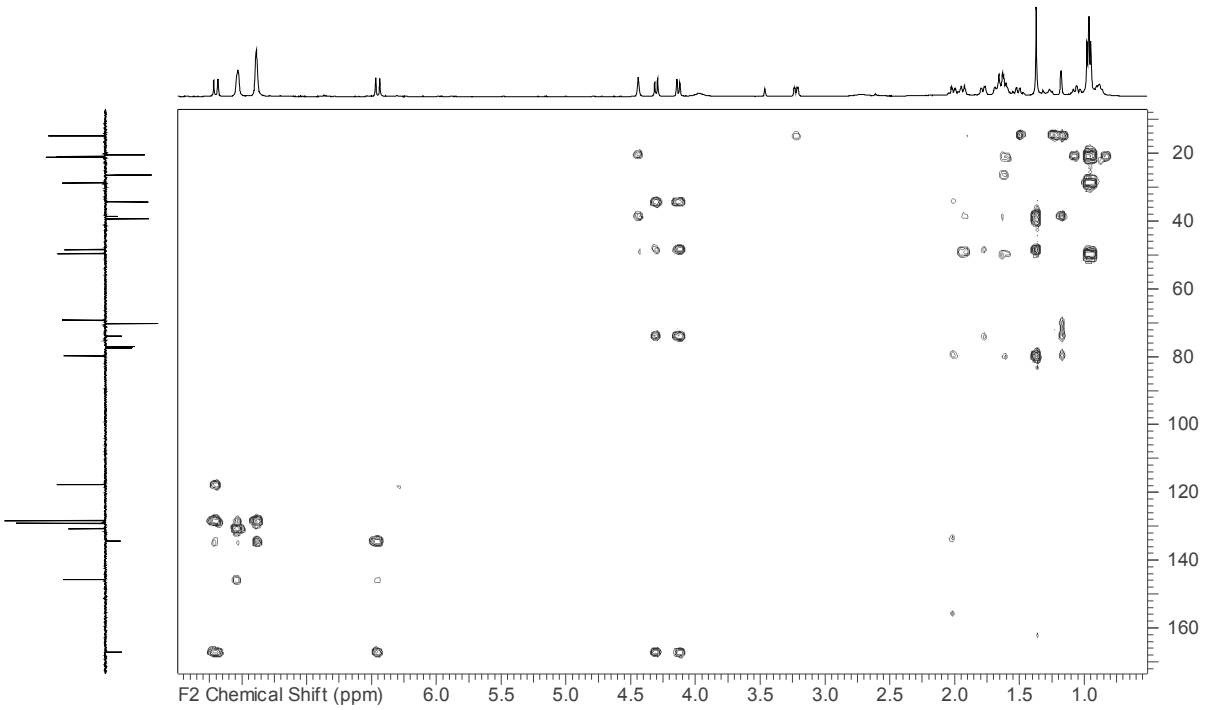


Figure S69. HMBC spectrum of compound **16** (500 MHz, $CDCl_3$)

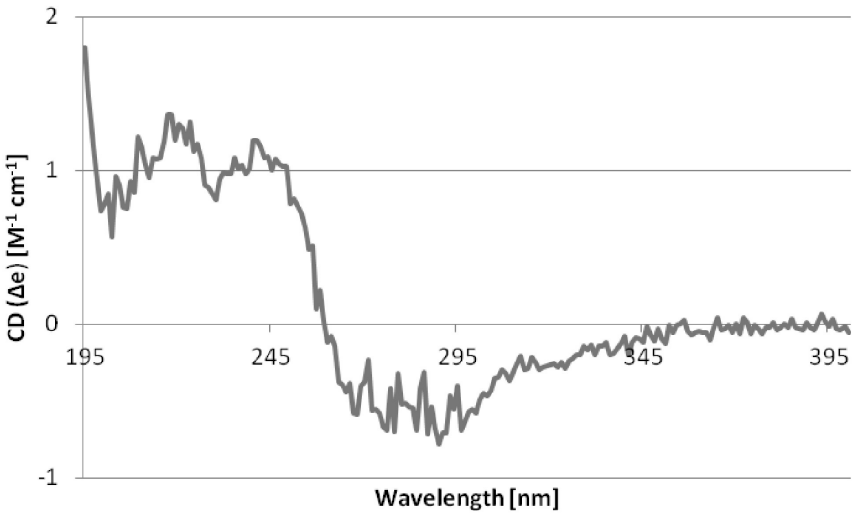
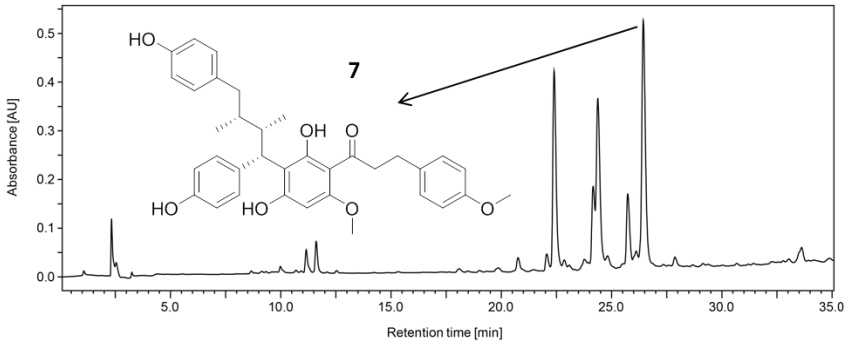


Figure S70. ECD spectrum of compound **16** in MeOH (0.2 mg/mL)

3.5. Flavonolignans from *Iryanthera megistocarpa* with Inhibitory Activity against Major Agricultural Pathogens

Justine Ramseyer, Barbara Thuerig, Maria De Mieri, Mengjie Zhu, Markus Neuburger, Hans-Jakob Schärer, Thomas Oberhänsli, Mahabir P. Gupta, Lucius Tamm, Matthias Hamburger, and Olivier Potterat

Results drafted as a manuscript



Iryanthera megistocarpa (Myristicaceae) leaves extracted with ethyl acetate showed significant activity against *Plasmopara viticola* (grapevine downy mildew), *Venturia inaequalis* (apple scab), and *Phytophthora infestans* (tomato and potato late blight) *in vitro*. The activity of the extract was then investigated on grapevine and tomato seedlings. Compared to non-treated controls, a formulation diluted at 1 mg/mL of extract reduced leaf surface infestation by 87% for *P. viticola* and by 68% for *P. infestans*. From the extract, two dihydrochalcones and eight flavonolignans including several stereoisomers were isolated. The two most active compounds showed MIC_{100} values $\leq$2.3 μg/mL against each tested pathogens.

Extraction of plant material, HPLC-microfractionation, preparative fractionation, and isolation of active compounds were performed by Mengjie Zhu under my supervision. Acquisition and interpretation of the data (except HR-MS) for structural elucidation together with Maria De Mieri, writing the manuscript draft, and preparing the figures were my contributions to this work.

Justine Fabienne Ramseyer

Flavonolignans from *Iryanthera megistocarpa* with Inhibitory Activity against Major Agricultural Pathogens

Justine Ramseyer,[†] Barbara Thuerig,[‡] Maria De Mieri,[†] Mengjie Zhu,[†] Markus Neuburger,[§] Hans-Jakob Schärer,[‡] Thomas Oberhänsli,[‡] Mahabir P. Gupta,[⊥] Lucius Tamm,[‡] Matthias Hamburger,[†] and Olivier Potterat*[,†]

[†]Division of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel, Klingelbergstrasse 50, 4056 Basel, Switzerland

[‡]Research Institute of Organic Agriculture FiBL, Department of Crop Sciences, Ackerstrasse 113, 5070 Frick, Switzerland

[§]Department of Chemistry, University of Basel, Mattenstrasse 24a, 4056 Basel, Switzerland

[⊥]Center for Pharmacognostic Research on Panamanian Flora, Faculty of Pharmacy, University of Panama, Panama City, Republic of Panama

ABSTRACT

An ethyl acetate extract of the leaves of *Iryanthera megistocarpa* showed significant inhibitory activity against *Plasmopara viticola* (grapevine downy mildew), *Venturia inaequalis* (apple scab), and *Phytophthora infestans* (tomato and potato late blight) with MIC_{100} values of 6.2, 23.2, and ≥125 μg/mL, respectively. On grapevine and tomato seedlings, a formulation of the extract diluted to 1 mg/mL of extract reduced leaf surface infestation by 87 and 68%, respectively, compared to non-treated controls. From the active extract, two dihydrochalcones and eight flavonolignans including several stereoisomers were isolated by a combination of chromatographic methods. Compounds **3** and **5** were the most active compounds with MIC_{100} ≤2.3 μg/mL against the three pathogens in vitro.

INTRODUCTION

Plant pathogens are an important threat to crops and the use of pesticides is hardly avoidable to control them. However, the deleterious effects on human health and environment of chemical pesticides have become a major concern.[1-5] Therefore, there is an increasing demand for new and safer products. Natural products, such as plant extracts, could afford some sustainable and environmentally friendly alternatives.[6-10] In the context of a search for safer substitutes of copper fungicides, an in-house library consisting of over 3000 extracts of plant and fungal origin (10 mg/mL in DMSO) was screened in vitro for inhibitory activity against grapevine downy mildew (*Plasmopara viticola* (Berk. & M. A. Curtis) Berl. & De Toni), apple scab (*Venturia inaequalis* (Cooke) G. Winter), and potato/tomato late blight (*Phytophthora infestans* (Mont.) de Bary).[11-12] An ethyl acetate extract from the leaves of an unspecified *Iryanthera* (Myristacaceae) species showed promising activity against the three investigated pathogens. Following this preliminary observation, leaves of *Iryanthera megistocarpa* A. H. Gentry were collected and extracted with ethyl acetate. The extract demonstrated significant activity against *P. viticola*, *V. inaequalis*, and *P. infestans*. The genus *Iryanthera* comprises about 20 species of trees and shrubs distributed from the northern half of South America to Panama.[13-15] The species *Iryanthera megistocarpa*, endemic to Panama, has not been phytochemically investigated up to now.[16-17]

Herein we report on the isolation and structure elucidation of two chalcones (**1** and **2**) and eight flavonolignans (**3-10**). These compounds possess significant inhibitory activity against the three aforementioned pathogens.

RESULTS AND DISCUSSION

Compound Isolation and Structure Elucidation. To identify the active constituents, a small amount of the ethyl acetate extract of *I. megistocarpa* leaves was submitted to HPLC-based activity profiling, a procedure combining microfractionation and bioactivity assessments.[12] The analysis allowed to correlate the activity with a group of UV active peaks eluting between 20 to 27.5 min (Figure S1, Supporting Information). For preparative isolation a larger amount of the extract was separated by column chromatography on silica gel to provide 11 fractions (Frs A-K) based on their TLC profile. Peaks detected in the active region of the extract chromatogram were mainly localized in Frs F, H, I, and J. Targeted isolation by a combination of preparative and semi-preparative HPLC afforded compounds **1**-**10**, corresponding to the major peaks of the extract UV trace (Figure 1).

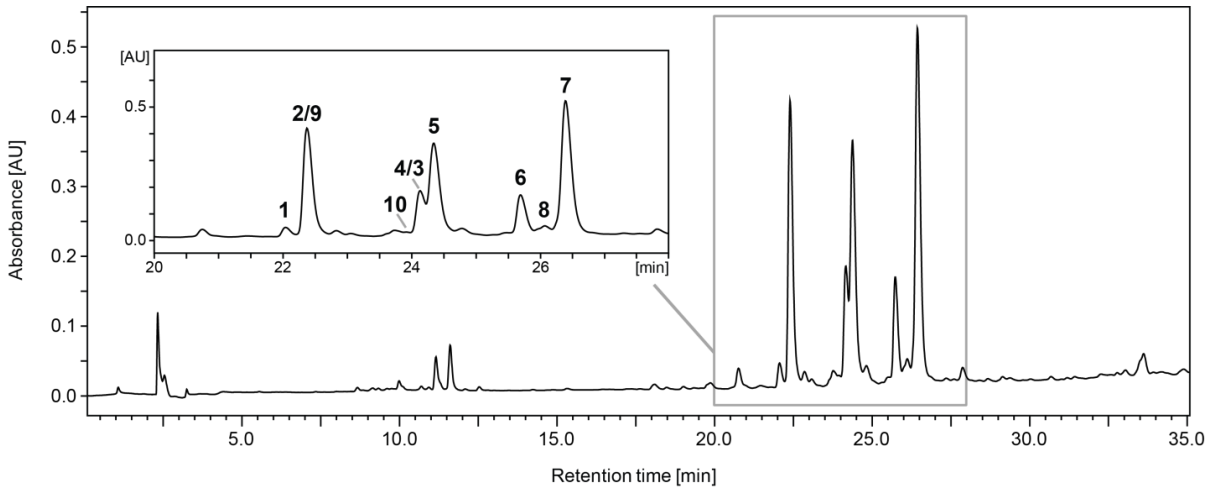
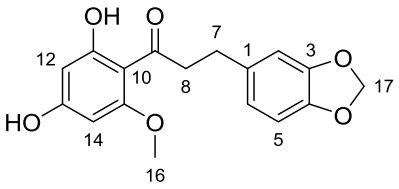
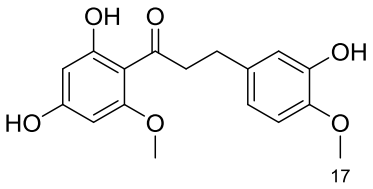


Figure 1. HPLC-PDA analysis of *Iryanthera megistocarpa* ethyl acetate extract. SunFire C_{18} column; A: water + 0.1% formic acid, B: acetonitrile + 0.1% formic acid; 5% to 100% B in 30 min, and 100% B for 5 min; detection 254 nm. Bolded numerals refer to isolated compounds.

Compounds **1** and **2** were identified by NMR spectroscopic data analysis and comparison with literature values as 2',4'-dihydroxy-6'-methoxy-3,4-methylenedioxydihydrochalcone (**1**)[18] and 2',4'-dihydroxy-4,6'-dimethoxydihydrochalcone (**2**)[18]. These compounds were previously isolated from different *Iryanthera* species.[19-25] Full ^{1}H and ^{13}C NMR spectroscopic assignments are provided as Supporting Information (Table S1).



1



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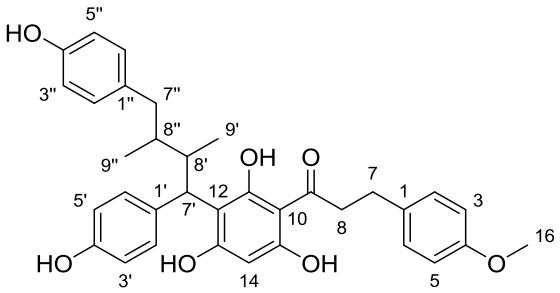
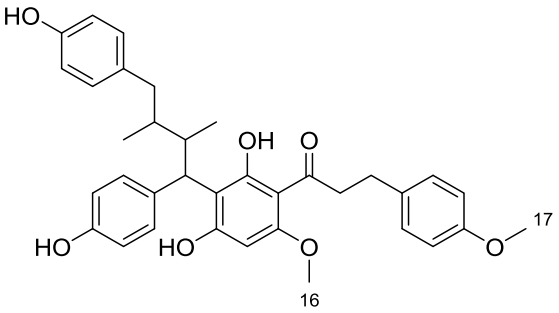


3 / 4 / 5



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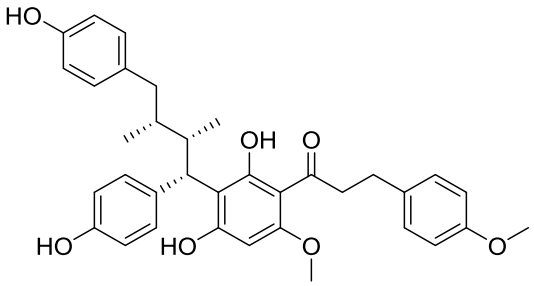

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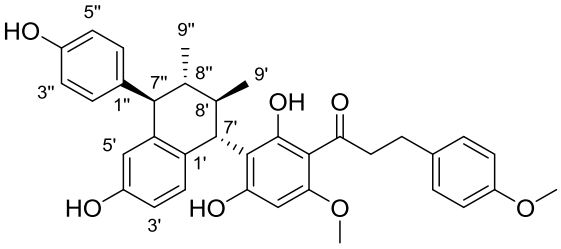

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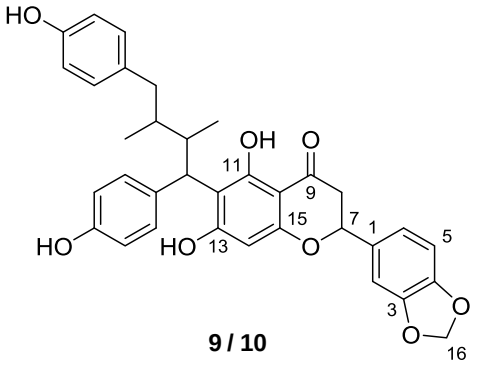

9 / 10

Compounds **3**, **4**, and **5** were obtained as brown oil and had an identical molecular formula, determined as $C_{34}H_{36}O_7$ from the HRESIMS $[M + H]^+$ ions at *m/z* 557.2537, 557.2536, and 557.2535, respectively (calcd $C_{34}H_{37}O_7^+$, 557.2534). By comparison of their NMR spectroscopic data (Table 1) and literature values, compounds **3**, **4**, and **5** were found to have the same planar structure as iryantherins G and H isolated from the fruits of *Iryanthera grandis*.[26] Some NMR shift differences were observed in the chain of the lignan moiety, indicating different stereoisomers.

Compounds **6** and **7**, isolated as light yellow oil, had the same molecular formula $C_{35}H_{38}O_7$ as established by the HRESIMS $[M + H]^+$ ions at same *m/z* 571.2691 (calcd $C_{35}H_{39}O_7^+$,

571.2690). NMR spectroscopic data (Table 2) and comparison with literature values revealed that they had the same planar structure as iryantherin B isolated from the bark of *Iryanthera laevis*,[19,27-28] and as iryantherins K and L isolated from *Iryanthera lancifolia*.[21] Some NMR shift differences observed in the chain of the lignan moiety indicated different stereoisomers.

Compound **8**, a pale yellow oil, gave a molecular formula of $C_{35}H_{36}O_7$ as deduced by the $[M + H]^+$ ion at *m/z* 569.2538 (calcd $C_{35}H_{37}O_7^+$, 569.2534) in the HRESIMS. The ^{13}C NMR spectrum showed the presence of signals for four methyls (including two methoxy groups), two methylenes, 16 methines (including twelve aromatic carbons), six oxygenated aromatic carbons (δ_C 154.0, 155.5, 157.5, 160.9/161.2, 164.6, and 165.2), one carbonyl (δ_C 204.0), and six quaternary carbons. Comparison of the NMR spectroscopic data (Table 2) with those of compounds **6** and **7** indicated that the dihydrochalcone moiety was identical and the difference concerned the lignan moiety. Cyclization occurred between the aliphatic carbon C-7'' (δ_C 53.0/53.1) and the aromatic carbon C-6' (δ_C 141.4) which was confirmed by the HMBC correlations of H-7'' (δ_H 3.51) to C-6' (δ_C 141.4) and of H-5' (δ_H 5.94) to C-7'' (δ_C 53.0/53.1). The relative configuration at C-7', C-8', C-7'', C-8'' was assigned by analysis of the *J* coupling of the tetrahydronaphthaline ring system as follows: the *J* couplings of 11.5 Hz shown by H-7' (δ_H 4.26 and 4.22) and H-7'' (δ_H 3.51) inferred their anti-periplanar orientation with respect to H-8' and H-8'', respectively. Key ROESY correlations H-7'$_\beta$ (δ_H 4.22/4.26) to H_3-9' (δ_H 0.82), and to H-8''$_\beta$ (δ_H 1.64); H_3-9' (δ_H 0.82) to H-8''$_\beta$ (δ_H 1.64); H-8'$_\alpha$ (δ_H 2.27) to H_3-9'' (δ_H 0.78), H-9'' (δ_H 0.78) to H-7''$_\alpha$ (δ_H 3.51), and H-7''$_\alpha$ (δ_H 3.51) to H-8'$_\alpha$ (δ_H 2.27), confirmed the cofacial orientation of H-7' and H-8'' and of H-8' and H-7'', arbitrarily assigned as beta and alpha, respectively.

Compounds **9** and **10** were isolated as yellow oils. Both had the molecular formula $C_{34}H_{32}O_8$ as determined by the HRESIMS $[M + H]^+$ ions at *m/z* 569.2173 and 569.2176, respectively (calcd $C_{34}H_{33}O_8^+$, 569.2170). According to the molecular formula and the NMR data (Table 3), compounds **9** and **10** possessed the same lignan moiety as **3**-**7** but differed from the latter compounds by the cyclization of the dihydrochalcone moiety to form a flavanone scaffold. The cyclization was in particular revealed by the lack of the methylene group at C-7, which was replaced by an oxygenated methine whose shifts were characteristic for the aryl substituted β position of a dihydrobenzopyranone (δ_H 5.34, δ_C 78.9). Further inspection of the NMR data, revealed a methylenedioxy group, correlating in the HMBC spectrum with two oxygenated sp^2 carbons at δ_C 147.6 and δ_C 147.5. The location of the latter substituent on the B ring of the flavanone was inferred through HMBC correlations with a three protons aromatic spin system (e.g. for **9**: H-2 (δ_H 7.09) to C-3 (δ_C 147.6) and H-5 (δ_H

6.97) to C-4 (δ_C 147.5)). The relative configuration of C-7 and C-8 was established from ^{1}H-^{1}H coupling constants and ROESY correlations. Thus, for compound **9**, the orientation of the aromatic ring attached at C-7 was determined as equatorial on the basis of the diaxial coupling of H-7$_{ax}$ (δ_H 5.34, d, J = 12.5 Hz) with H-8$_{ax}$ (δ_H 3.08, dd, J = 17.0/12.5 Hz). Conversely, for compound **10**, the orientation of the aromatic B ring was determined as axial by the absence of any diaxial coupling of H-7 (δ_H 5.37, br m) with H$_a$-8 (δ_H 3.19, br m) or with H$_b$-8 (δ_H 2.62, br d, J = 16.5 Hz), thus establishing H-7 in equatorial orientation.

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds 3-5 (DMSO-d_6; 500 MHz for ^{1}H, 125 MHz for ^{13}C; δ in ppm)

	3[a]		**4**[a]		**5**[a]	
position	δ_H, (J in Hz)	δ_C, type	δ_H, (J in Hz)	δ_C, type	δ_H, (J in Hz)	δ_C, type
1	-	133.6, C	-	133.7, C	-	133.5/133.6, C
2, 6	7.14, br m	129.3, CH	7.14, d (7.0)	129.3, CH	7.12/7.13, d (8.0)	129.3, CH
3, 5	6.83, d (7.6)	113.8, CH	6.81, d (7.0)	113.7, CH	6.82, d[c] (8.0)	113.7, CH
4	-	157.5, C	-	157.4, C	-	157.4, C
7	2.84, br m	29.6/29.7, CH_2	2.83, br m	29.7, CH_2	2.80/2.83, t (7.0)	29.5/29.6, CH_2
8	3.24, t (7.6)	45.5/45.6, CH_2	3.27, br m	45.4/45.5, CH_2	3.23/3.24, t (7.0)	45.5, CH_2
9	-	204.2/204.4, C	-	203.7, C	-	204.1/204.4, C
10	-	103.5/103.6[b], C	-	103.7/104.4[b], C	-	103.3/103.5, C
11	-	163.3/163.4[b], C	-	163.3/163.8[b], C	-	163.5/164.3, C
12	-	108.9/109.1[b], C	-	107.9/108.5[b], C	-	109.7/109.8, C
13	-	162.0[b], C	-	n.d.[d], C	-	162.3/163.0, C
14	5.98, s	94.2/94.7, CH	5.93/6.00, s	94.9/95.4, CH	5.86/6.00, s	94.1/94.6, CH
15	-	159.7/159.8[b], C	-	n.d.[d], C	-	159.6/159.8, C
16	3.70, s	55.0, CH_3	3.70, s	55.0, CH_3	3.71, s	55.0, CH_3
1’	-	135.6/135.8, C	-	135.4, C	-	134.4/134.6, C
2’, 6’	7.25, d (8.2)	129.4, CH	7.37, d (7.0)	129.6, CH	7.06/7.08, d (8.0)	129.5/129.6, CH
3’, 5’	6.59, d (8.2)	114.5, CH	6.65, d[c] (7.0)	114.5, CH	6.54, d (8.0)	114.2, CH
4’	-	155.0, C	-	154.9, C	-	154.8, C
7’	4.04, m	44.2/44.5, CH	4.40/4.45, d (11.0)	42.4/42.8, CH	4.18/4.25, d (11.7)	44.2/44.5, CH
8’	2.92, m	35.2/36.5, CH	2.92/2.97, br m	37.3/37.8, CH	2.89/2.97, m	33.3/33.4, CH
9’	0.65, d (6.7)	12.4, CH_3	0.73, br m[c]	12.3, CH_3	0.63, d (6.7)	11.3/11.5, CH_3
1’’	-	131.6/131.7, C	-	132.0, C	-	131.4/131.5, C
2’’, 6’’	6.86, br m	129.6, CH	6.66, d[c] (7.0)	129.5, CH	6.82, d[c] (8.3)	129.7, CH
3’’, 5’’	6.65, d (7.5)	114.8/114.9, CH	6.58, d (7.0)	114.8, CH	6.66, d (8.3)	114.8, CH
4’’	-	155.1, C	-	155.1, C	-	155.1, C
7’’	2.32, dd (13.0, 8.8)	41.5/41.8, CH_2	1.85, dd (12.0/12.0)	34.3, CH_2	2.32, dd (13.0, 8.8)	41.1/41.3, CH_2
	2.44, m		2.74, br m		2.44, m	
8’’	1.73, m	36.0/36.1, CH	1.61, br m	35.8, CH	1.72, m	34.8/35.0, CH
9’’	0.60, br m	11.9, CH_3	0.70, br m[c]	18.4, CH_3	0.66/0.69, d (6.7)	12.3/12.5, CH_3

[a]For some ^{1}H and ^{13}C NMR signals which are double in the spectra, both values are listed.
[b]Assigned on the basis of ACDLabs predicitions.
[c]Overlapping signals.
[d]n.d. = not detected.

Table 2. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds 6-8 (DMSO-d_6; 500 MHz for ^{1}H, 125 MHz for ^{13}C; δ in ppm)

	6[a]		**7**[a]			**8**[a]	
position	δ_H, (J in Hz)	δ_C, type	δ_H, (J in Hz)	δ_C, type	position	δ_H, (J in Hz)	δ_C, type
1	-	133.4, C	-	133.4, C	1	-	133.4, C
2, 6	7.13, d (7.0)	129.3, CH	7.11/7.12, d (8.2)	129.3, CH	2, 6	7.13/7.18, br d (8.0)	129.3, CH
3, 5	6.82, d (7.0)	113.8, CH	6.82, d[c] (8.2)	113.8, CH	3, 5	6.83/6.85, br d (8.0)	113.8, CH
4	-	157.5, C	-	157.5, C	4	-	157.5, C
7	2.82, br m	29.6/29.8, CH_2	2.80, m	29.6, CH_2	7	2.80/2.88, t (6.5)	29.6, CH_2
8	3.17, m	45.5/45.6, CH_2	3.16, m	45.5/45.6, CH_2	8	3.19/3.27, t (6.5)	45.6, CH_2
9	-	203.9/204.1, C	-	203.9/204.1, C	9	-	204.0, C
10	-	103.9[b], C	-	103.8/103.9[b], C	10	-	103.4/104.4[b], C
11	-	165.0[b], C	-	164.9[b], C	11	-	165.2[b], C
12	-	110.3[b], C	-	111.0/111.1[b], C	12	-	110.8/111.1[b], C
13	-	164.0[b], C	-	164.1[b], C	13	-	164.6[b], C
14	6.05, s	90.8/91.2, CH	5.90/6.07, s	90.7/91.1, CH	14	5.99/6.18, br s	90.3/91.7, CH
15	-	160.6/160.7, C	-	160.5/160.6, C	15	-	160.9/161.2, C
16	3.74, br s	55.5, CH_3	3.72/3.75, s	55.4/55.5, CH_3	16	3.79/3.83, s	55.5/55.6, CH_3
17	3.70, s	55.0, CH_3	3.71, s	55.0, CH_3	17	3.72, s	54.6, CH_3
1’	-	135.2/135.7, C	-	134.1/134.3, C	1’	-	130.6, C
2’, 6’	7.24, d (7.5)	129.3, CH	7.08, d (8.3)	129.5/129.6, CH	2’	6.42, d (8.5)	127.0/127.2, CH
3’, 5’	6.58, d (7.5)	114.5, CH	6.54, d (8.3)	114.3, CH	3’	6.29, dd (8.5, 2.2)	112.8, CH
4’	-	155.0, C	-	154.9, C	4’	-	154.0, C
					5’	5.94, d (2.2)	115.2, CH
					6’	-	141.4, C
7’	4.08, d (10.7)	44.2/44.5, CH	4.23/4.27, d (12.0)	42.0/42.4, CH	7’	4.22/4.26, d (11.5), ax	40.8/41.3, CH
8’	2.94, m	35.0/36.4, CH	2.92/2.97, m	33.3/33.4, CH	8’	2.27, br m, ax	37.3/37.6, CH
9’	0.65, d (6.6)	12.3, CH_3	0.63, d (6.4)	11.4/11.5, CH_3	9’	0.82, d (6.7)	17.9/18.0, CH_3
1’’	-	131.6, C	-	131.4/131.5, C	1’’	-	136.5, C
2’’, 6’’	6.85, br m	129.6, CH	6.82, d[c] (8.2)	129.7, CH	2’’, 6’’	6.94, d (8.5)	130.2, CH
3’’, 5’’	6.64, d (6.7)	114.9, CH	6.66, d (8.2)	114.8, CH	3’’, 5’’	6.74, d (8.5)	115.1, CH
4’’	-	155.1, C	-	155.2, C	4’’	-	155.5, C
7’’	2.32, dd (13.5, 8.9)	41.5/41.8, CH_2	2.35, m	41.1/41.3, CH_2	7’’	3.51, d (11.5), ax	53.0/53.1, CH
	2.43, br m		2.39, m				
8’’	1.72, m	36.0, CH	1.73, br m	34.8/35.0, CH	8’’	1.64, br m, ax	43.1, CH
9’’	0.60, br m	11.9, CH_3	0.67/0.69, d (6.7)	12.3/12.4, CH_3	9’’	0.78, d (6.5)	17.8, CH_3

[a]For some ^{1}H and ^{13}C NMR signals which are double in the spectra, both values are listed.

[b]Assigned on the basis of ACDLabs predictions.

[c]Overlapping signals.

Table 3. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds 9 and 10 (DMSO-d_6; 500 MHz for ^{1}H, 125 MHz for ^{13}C; δ in ppm)

	9		10	
position	δ_H, (J in Hz)	δ_C, type	δ_H, (J in Hz)	δ_C, type
1	-	133.3, C	-	132.8, C
2	7.09, br s	106.8, CH	7.06, br s	107.3, CH
3	-	147.6, C	-	147.4, C
4	-	147.5, C	-	147.4, C
5	6.97, m[a]	108.2, CH	6.90, d (8.0)	108.1, CH
6	6.96, m[a]	120.3, CH	6.93, dd (8.0, 1.2)	120.7, CH
7	5.34, d (12.5), ax	78.9, CH	5.37, br m, eq[d]	78.3, CH
8	2.61, m[a], eq	43.1, CH_2	2.62, br d (16.5), ax[d]	42.4, CH
	3.08, dd (17.0, 12.5), ax		3.19, br m, eq[d]	
9	-	n.d.[b], C	-	195.3, C
10	-	101.4[c], C	-	101.1[c], C
11	-	159.6[c], C	-	160.6[c], C
12	-	110.6[c], C	-	110.8[c], C
13	-	167.3[c], C	-	161.6[c], C
14	5.93, s	96.2, CH	5.95, s	95.3, CH
15	-	161.3[c], C	-	160.2[c], C
16	6.06, s	101.3, CH_2	6.02, s	101.2, CH_2
1’	-	135.0, C	-	135.5, C
2’, 6’	7.16, d (7.9)	129.5, CH	7.25, br d (8.0)	129.3, CH
3’, 5’	6.58, d (7.9)	114.4, CH	6.59, d (8.0)	114.6, CH
4’	-	155.0, C	-	155.1, C
7’	4.15, d (9.5)	43.9, CH	4.03, d (9.5)	44.3, CH
8’	2.65, m[a]	36.5, CH	2.93, br m	35.4, CH
9’	0.60, d (6.7)	12.7, CH_3	0.65, d (6.7)	12.2, CH_3
1’’	-	131.5, C	-	132.0
2’’, 6’’	6.87, d (7.5)	129.7, CH	6.86, br m	129.7, CH
3’’, 5’’	6.66, d (7.5)	115.5, CH	6.63, d (8.2)	114.8, CH
4’’	-	155.3, C	-	155.1, C
7’’	2.23, m	41.6, CH_2	2.32, br m	41.5, CH_2
			2.45, br m	
8’’	1.64, m	36.3, CH	1.75, br m	36.1, CH
9’’	0.55, m	11.4, CH_3	0.62, d (5.5)	12.3, CH_3

[a] Overlapping signals.
[b] n. d. = not detected.
[c] Assigned based on ACDLabs predictions.
[d] Determined by ROESY correlations.

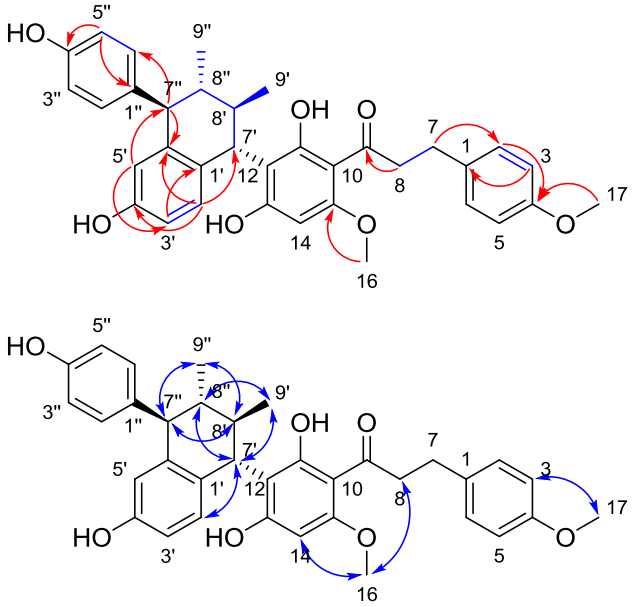

Figure 2. Key COSY (blue bonds), HMBC (red arrows), and ROESY correlations (blue arrows) of compound **8**.

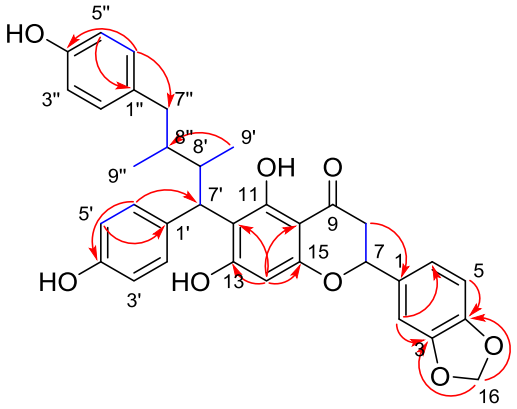

Figure 3. Key COSY (blue bonds) and HMBC (red arrows) correlations of compound **9**.

Silva *et al*. reported the relative configuration of the C-7',C-8',C-8'' chain for the structures of compounds **3-7** based on NOESY correlations.[21,26] However, after conformation calculations with Macromodel 9.8 software (Schrödinger LLC) employing the OPLS 2005 force field in H_2O (data not shown), the observed ROESY correlations were shown to be indeed compatible with each stereoisomer and therefore do not enable a reliable discrimination. Moreover, comparison of the ^{1}H-NMR spectra with reported literature values did not allow to assign the compounds to previously reported stereoisomers due to peak broadening and some double signals observed in our ^{1}H and ^{13}C experiments for compounds

3-**8**. These double signals are assumed to be due to the presence of different conformations in equilibrium. The NMR spectra are available as Supporting Information. In NMR experiments at increased temperatures (up to 60°C) the corresponding signals converged, but did not coalesce (data not shown). In addition, the conformational analysis of compound **3** of the possible diastereoisomer 7'*R*,8'*R*,8''*R* (Figure 4) showed two conformations at low energy (up to 2.7 kJ/mol), which may be responsible for the observed peak broadening and the double signals.

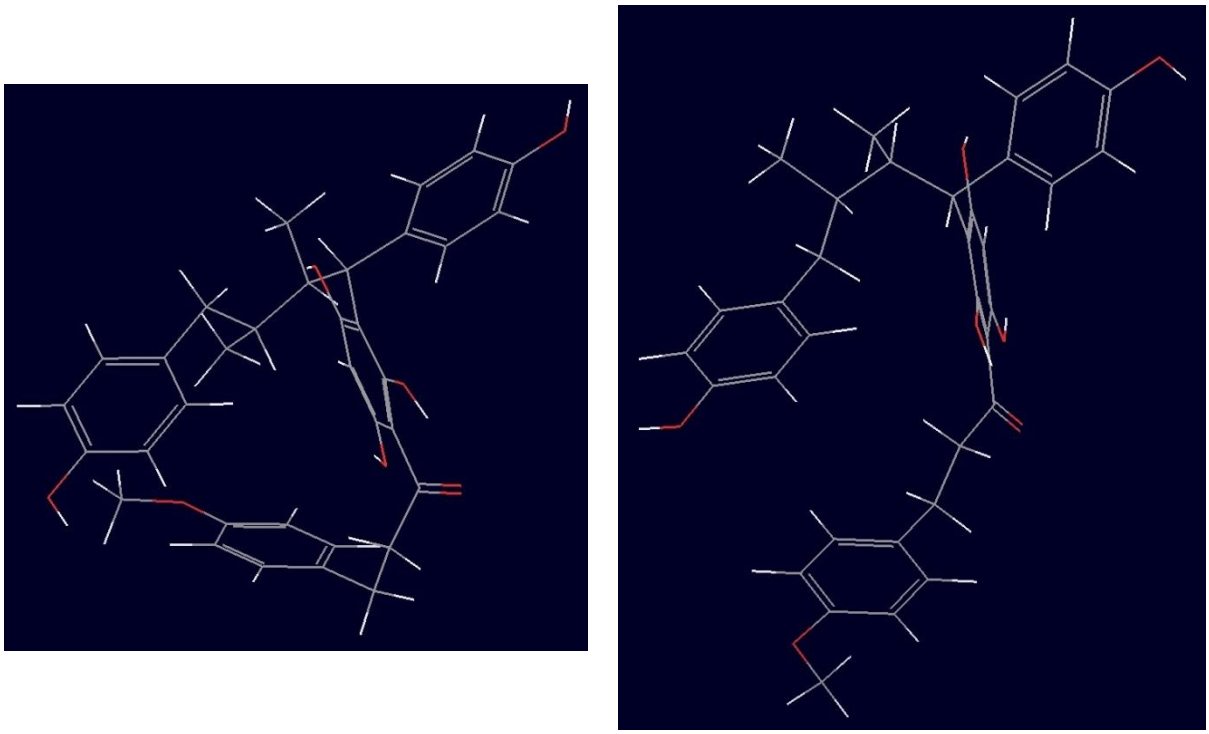

Figure 4. Two calculated conformations of compound **3** for the possible diastereoisomer 7'*R*,8'*R*,8''*R* (up to 2.7 kJ/mol).

Attempts to assign the configuration of the C-7',C-8',C-8'' chain in compounds **3**-**10** by electronic circular dichroism (ECD) measurements in combination with quantum calculations with two different algorithms (B3LYP and cam-B3LYP) remained also unsuccessful (data not shown).

Suitable plates were obtained for compound **7** in a mixture of toluene and acetone. X-ray analysis with Ga K_α radiation enabled to assign the relative configuration of the C-7',C-8',C-8'' chain (Figure 5). The data also confirmed the position of the methoxy group at C-15, which was assigned by a weak NOESY correlation in NMR (data not shown). However, the data set was not of sufficient quality for publication in a X-ray database. Convergence could

not be reached due to the lack of enough observed reflection data. The opposite optical rotation of compound **7** (+36) and iryantherin K^{21} (-36) suggests that these compounds are enantiomers.

Attempts to crystallize compounds **3-6** and **8-10** in several solvent mixtures remained unsuccessful.

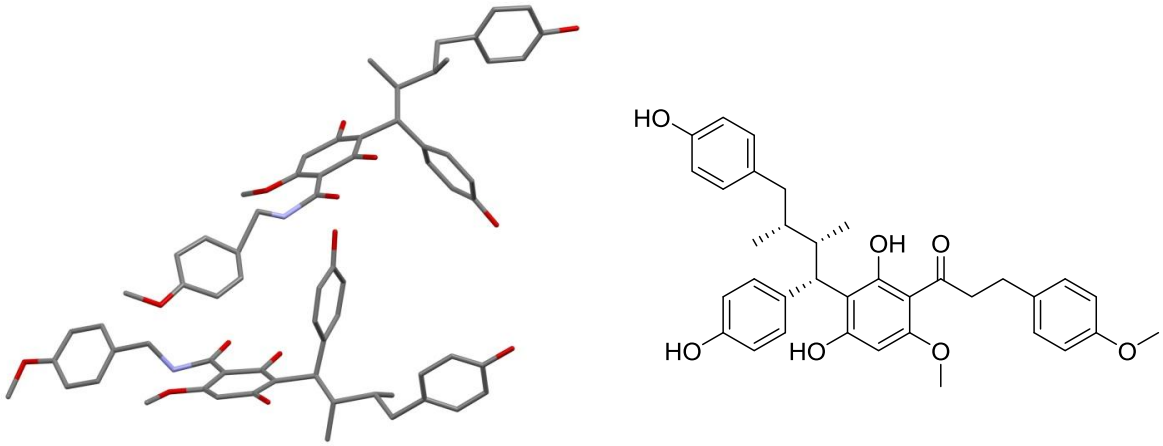

Figure 5. X-ray structure of compound **7**. There are two molecules present in the asymmetric unit that are not related by symmetry. Both molecules have the same stereochemistry, but show slightly different conformations.

Antifungal Activity. In vitro minimal inhibitory concentrations (MIC_{100}) of the crude plant extract against *P. viticola*, *V. inaequalis*, and *P. infestans* were determined in three independent experiments and means were 6.2, 23.2, and ≥125 μg/mL, respectively (Table 4). MIC_{100} of pure compounds were also assessed, except for **4** and **9** which were not available in sufficient amounts (Table 4). The two most potent compounds (**3** and **5**) showed mean MIC_{100} ≤2.3 μg/mL against all three pathogens. Most of the pure compounds were significantly more active than the crude extract. This confirmed that the dihydrochalcones and flavonolignans are responsible for the activity of *I. megistocarpa* extract. It must be pointed out that a broad variation of the potency was observed between independent in vitro assays for some compounds. Such variation was not observed for controls or compounds from other sources tested in parallel, which means that the discrepancy was not resulting from the testing conditions. In addition, solubility did not appear to be an issue since no precipitation was observed after compound addition into the test wells. Further investigations should be carried out to identify the reasons for these variations.

In a next step, the activity of the extract was assessed on grapevine (*P. viticola*) and tomato (*P. infestans*) seedlings (Figure 6). An emulsifiable concentrate formulation of the crude extract (60 mg/g) was developed to enhance the solubility (IM-EC). Two concentrations were tested, namely 1 mg/mL and 0.5 mg/mL of extract. The effect of the blank formulation was also assessed at corresponding concentration of additives. At a concentration of 1 mg/mL, the efficacies were of 87% on grapevine and of 68% on tomato seedlings. The values were only slightly lower at 0.5 mg/mL of extract, namely 79% on grapevine and 60% on tomato. The efficacies were expressed, as the lowering of infected leaf surface in the treated set compared to the non-treated control set of seedlings (grapevine disease severity of 93 ± 8% and tomato disease severity of 64 ± 12%). The tests on apple plants are ongoing. Some assays with selected pure compounds have been also scheduled.

Approximately ten species of the genus *Iryanthera* have been phytochemically investigated up to now, and dihydrochalcones and flavonolignans have been reported as characteristic constituents of these plants.[19-22,24-46] A few species have been biologically investigated. Extracts from *I. laevis*,[19] *I. tricornis*,[47] and *I. megistophylla*[34,47] showed antimicrobial activities. No antifungal properties have been so far reported for this group of flavonolignans.

In conclusion, dihydrochalcones, flavonolignans, and extracts from *Iryanthera* species may have a potential for the development of new natural pesticides. As a further step, toxicological studies should be performed to insure the safety of these barely investigated compounds. Furthermore, the activity will need to be confirmed in studies under field conditions.

Table 4. In Vitro Minimal Inhibitory Concentrations (MIC_{100}) of *Iryanthera megistocarpa* Extract and Selected Constituents against *Plasmopara viticola*, *Venturia inaequalis*, and *Phytophthora infestans*

	Plasmopara viticola			
	MIC_{100} / Strong inhibition[a] [µg/mL]			
compound	Exp. 1[b]	Exp. 2	Exp. 3	mean MIC_{100}[c]
1[d]	12.5	50 / 25	33	27.4
2	3.1	1.6	66 / 33	6.9
3	0.2	1.6	2.5 / 1.3	0.9
5	0.4	1.6 / 0.8	2.5 / 1.3	1.2
6	1.6	6.3 / 1.6	10.0 / 2.5	4.6
7	6.3 / 3.1	25 / 3.1	2.5 / 1.3	7.3
8	6.3 / 3.1	>25 / 3.1	2.5	-
10	100	no inhibition	2.5 / 1.3	-
extract	3.9	7.8 / 3.9	8 / 4	6.2

	Venturia inaequalis			
	MIC_{100} / Strong inhibition[e] [µg/mL]			
compound	Exp. 1[b]	Exp. 2	Exp. 3	mean MIC_{100}[c]
1[d]	50 / 25	50	66	54.8
2	50 / 12.5	no inhibition	no inhibition	-
3	0.4	3.1	1.3 / 0.7	1.2
5	0.4	1.6	0.7	0.7
6	25 / 1.6	3.1	3 / 1.3	6.2
7	25 / 6.3	1.6	10 / 1.3	7.3
8	100.0 / 25	3.1	10 / 2.5	14.6
10	50 / 25	6.3	20 / 5	18.4
extract	125 / 31	6.3 / 3.1	16	23.2

	Phytophthora infestans			
	MIC_{100} / Strong inhibition[f] [µg/mL]			
compound	Exp. 1[b]	Exp. 2	Exp. 3	mean MIC_{100}[c]
1[d]	100	50	66	69.1
2	no inhibition	no inhibition	66	-
3	1.6	6.3 / 3.1	1.3	2.3
5	3.1 / 1.6	3.1	1.3	2.3
6	no inhibition	no inhibition	20	-
7	no inhibition	no inhibition	40	-
8	no inhibition	no inhibition	40	-
10	no inhibition	no inhibition	80 / 40	-
extract	no inhibition	250 / 125	125	-

[a]Less than ten active zoospores (<0.1%).
[b]Independent experiments.
[c]Data $\log_2$-transformed to calculate mean and retransformed to linear scale.
[d]**4** and **9** were not tested due to the insufficient amounts available.
[e]Short germ tubes (approx. 1 to 3 lengths of conidia) or 1 to 5 conidia with long germ tubes (<0.1%).
[f]Short germ tubes (approx. 1 to 3 lengths of sporangia) or 1 to 5 sporangia with long germ tubes (<0.1%).

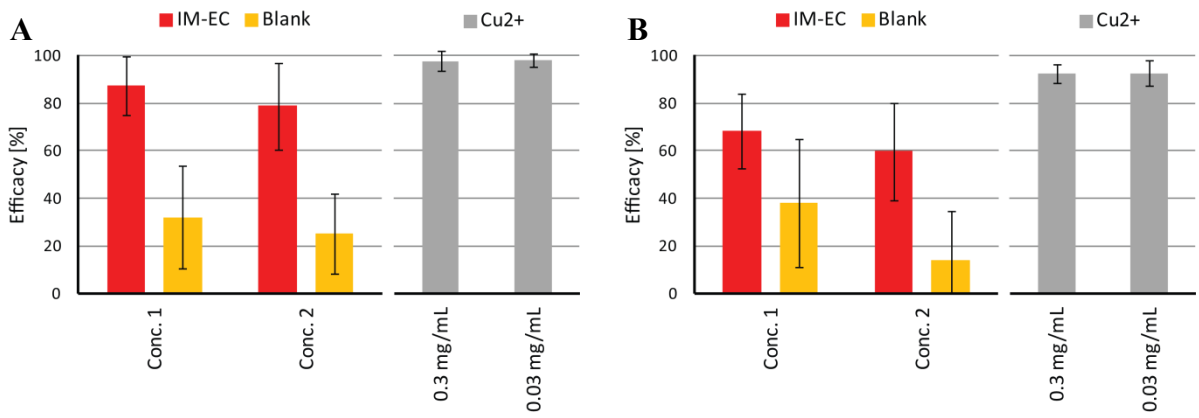

Figure 6. Efficacy of a formulated *Iryanthera megistocarpa* extract (IM-EC), blank formulation (Blank), and a copper control (Cu^{2+}) against *Plasmopara viticola* on grapevine seedlings (**A**) and *Phytophthora infestans* on tomato seedlings (**B**) under semicontrolled conditions. IM-EC contained 6% of extract and 94% additives. IM-EC and Blank were tested at two concentrations ["Conc. 1": 16.6 mg/mL IM-EC (1 mg/mL of extract) or 15.6 mg/mL of formulation additives (Blank); "Conc. 2": 8.3 mg/mL IM-EC (0.5 mg/mL of extract) or 7.8 mg/mL of formulation additives (Blank)]. The disease severity in the control was 93 ± 8% for *P. viticola* (**A**) and 64 ± 12 for *P. infestans* (**B**). The figure shows means and standard deviations of one experiment (n = 6).

EXPERIMENTAL SECTION

General Experimental Procedures. Formic acid, sulfuric acid, and solvents were obtained from Scharlau (Scharlab S. L.) or from Macron Fine Chemicals (Avantor Performance Materials). HPLC-grade solvents and ultrapure water from a MilliQ water purification system (Merck Millipore) were used for HPLC. For extraction and preparative separation, technical grade solvents were used after distillation. DMSO-d_6 was purchased from Armar AG.

Silica gel 60 F_{254} coated aluminum TLC plates, and silica gel (0.063-0.200 mm) for open column chromatography were obtained from Merck KGaA. TLC plates were visualized under UV-light and by spraying with 1% vanilin (Roth GmbH + Co) in EtOH, followed by 10% sulfuric acid in EtOH, and heating at 110°C.

HPLC-PDA-ESIMS analyses were performed on a LC-MS 8030 system (Shimadzu) using a SunFire C_{18} (3.5 µm, 150 x 3.0 mm i.d.) column equipped with a guard column (10 mm x 3.0 mm i.d.) (Waters). LabSolutions software (Shimadzu) was used for data acquisition and processing.

Semi-preparative HPLC was performed on an Agilent 1100 Series instrument equipped with a PDA detector. A SunFire C_{18} (5 µm, 150 x 10 mm i.d.) column with a guard column (10 mm x 10 mm i.d.) (Waters) was used. Data acquisition and processing were performed using ChemStation software (Agilent Technologies).

Preparative HPLC was carried out on a Puriflash 4100 system (Interchim), or on a system consisting of Shimadzu LC-8A binary pumps and an Agilent 1200 PDA detector. Separations were performed on a SunFire C_{18} (5 µm, 150 x 30 mm i.d.) column with guard column (10 mm x 20 mm i.d.) (Waters).

HRESIMS data were recorded in positive ion mode on an Agilent 1290 Infinity system with an Agilent 6540 UHD Accurate-Mass Quadrupole Time-of-Flight detector. Optical rotations were measured at a concentration of 1 mg/mL in MeOH on a P-2000 Digital Polarimeter (Jasco) equipped with a sodium lamp (589 nm) and a 10 cm temperature-controlled microcell. UV and ECD spectra were recorded, at a concentration of 0.2 mg/mL in MeOH, on a Chirascan CD spectrometer with 1 mm path precision cells 110 QS (Hellma Analytics). NMR spectra were recorded on a Bruker AVANCE III 500 MHz spectrometer operating at 500 MHz for ^{1}H and at 125 MHz for ^{13}C. The instrument was equipped with a 1 mm TXI microprobe operated at 18 °C, or a 5 mm BBO probe at 23 °C (Bruker Biospin). Chemical shifts are reported as δ values (ppm), with residual solvent signal as internal reference, *J* in Hz. Standard pulse sequences from Topspin 2.1 software package were used.

Plant Material. Leaves of *Iryanthera megistocarpa* were collected in October 2015 on the road from Llano to Cartí (9°17'18'' N, 78°58'09'' W, 361 m a.s.l.), Panama, by CIFLORPAN (Center for Pharmacognostic Research on Panamanian Flora); Panamanian collection number FLORPAN 8849. A voucher specimen is deposited at the Herbarium of the University of Panama (PMA). The taxonomic identity was confirmed by Alex Espinosa, botanist at CIFLORPAN. The material was air-dried in Panama. A voucher specimen (no. 960) is also available at the Division of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel.

Micro-fractionation of Extract for Activity Profiling. Extraction was performed by Accelerated Solvent Extraction (ASE) (ASE 200, Dionex) sequentially with petroleum ether, ethyl acetate, and MeOH (3 cycles of 5 min each at 70°C and 120 bars). 3.7 g of cryomilled leaves yielded 219.7 mg of ethyl acetate extract after evaporation under reduced pressure.

Micro-fractionation was performed on a semi-preparative HPLC system connected to a FC 204 fraction collector (Gilson). The extract was dissolved in DMSO at a concentration of 25 mg/mL. Two injections of 400 µL were performed (20 mg of extract in total) with UV

detection at 254 nm. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). Flow rate was 4.0 mL/min. A gradient of 5 to 100 % B in 30 min was applied, followed by isocratic conditions of 100 % B for 5 min. Micro-fractions were collected every 90 sec from 2 to 35 min (22 fractions pro injection). After removal of the eluent in a Genevac EZ-2 (SP Scientific) evaporator, the corresponding fractions from the two separations, redissolved in 300 µL of methanol, were combined in a 96-deep-well plate and re-dried in the Genevac EZ-2.

Extraction and Isolation. 250 g of cryomilled leaves were mixed with sea sand and percolated at room temperature with ethyl acetate (6.2 L) to afford, after evaporation under reduced pressure, 19.9 g of extract.

A portion of the ethyl acetate extract (19.4 g) was dissolved in ethyl acetate and adsorbed on ca. 50 g of silica gel. The dried powder was then loaded onto an open column filled with silica gel (65 cm x 5 cm i.d.). Elution was performed with a step gradient of *n*-hexane/ethyl acetate [10:0 (4.0 L), 95:5 (2.0 L), 90:10 (2.0 L), 80:20 (2.0 L), 70:30 (2.0 L), 60:40 (2.0 L), 50:50 (3.0 L), 25:75 (2.0 L), and 0:100 (2.5 L)] at a flow rate of approx. 25 mL/min to afford 11 fractions (Frs A-K) based on TLC analysis.

A portion (650 mg) of Fraction F (1970 mg) was submitted to preparative HPLC (Puriflash system) with 45% acetonitrile isocratic at a flow rate of 25 mL/min. Repeated injections afforded compounds **1** (15.3 mg, t_R 31.0 min) and **2** (205.3 mg, t_R 32.5 min).

Compounds **8** (11.8 mg, t_R 42.9 min), **9** (1.4 mg, t_R 17.5 min), and **10** (4.8 mg, t_R 28.4 min) were isolated from a portion (670 mg) of Fraction H (720 mg) by preparative HPLC (Shimadzu LC-8A system) with two isocratic steps at a flow rate of 20 mL/min: 54% acetonitrile for 37 min, followed by 70% for 10 min.

Purification of compounds **6** (22.7 mg, t_R 22.0 min) and **7** (88.5 mg, t_R 25.0 min) was performed by preparative HPLC (Puriflash system) on a portion (250 mg) of Fraction I (3120 mg) with 60% acetonitrile isocratic at a flow rate of 25 mL/min

A portion (200 mg) of Fraction J (2590 mg) was separated by preparative HPLC (Puriflash system) using two isocratic steps at a flow rate of 25 mL/min: 65% acetonitrile for 27 min followed by 65% for 15 min to give compounds **5** (47.4 mg, t_R 22.0 min), as well as **6** (5.5 mg, t_R 33.5 min) and **7** (18.3 mg, t_R 36.5 min) already isolated from Fraction I. Further purification of a subfraction (43.8 mg) by semi-preparative HPLC using isocratic elution with 70% methanol at a flow rate of 4 mL/min afforded compounds **3** (18.4 mg, t_R 20.7 min) and **4** (3.7 mg, t_R 25.3 min).

HPLC-PDA-ESIMS Analysis. Analyses were performed on a LC-MS 8030 system (Shimadzu) using a SunFire C_{18} (3.5 μm, 150 x 3.0 mm i.d.) column equipped with a guard column (10 mm x 3.0 mm i.d.) (Waters). The software for data acquisition and processing was LabSolutions (Shimadzu). UV and mass detection ranges were 190 to 600 nm and *m/z* 160-1500, respectively. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). A gradient of 5 to 100% B in 30 min was applied, followed by 100% B for 5 min. The flow rate was 0.4 mL/min. The samples were dissolved in DMSO (extract and fractions: 5 mg/mL; pure compounds 0.5 mg/mL) and 5 μL were injected. Compounds were identified in the extract or fractions by comparison of their ESIMS data and their retention times with those of the purified compounds.

2',4'-dihydroxy-6'-methoxy-3,4-methylenedioxydihydrochalcone (**1**): light yellow amorphous solid; $[\alpha]^{25}_{D}$ 3 (*c* 0.1, MeOH); UV λ_{max} (MeOH) (log ε) 228 (sh) (4.25), 289 (4.40) nm; ^{1}H and ^{13}C NMR, see Table S1, Supporting information; HRESIMS *m/z* 317.1023 $[M + H]^+$ (calcd for $C_{17}H_{17}O_6^+$, 317.1020).

2',4'-dihydroxy-4,6'-dimethoxydihydrochalcone (**2**): light yellow amorphous solid; $[\alpha]^{25}_{D}$ -1 (*c* 0.1, MeOH); UV λ_{max} (MeOH) (log ε) 224 (4.45), 286 (4.40) nm; ^{1}H and ^{13}C NMR, see Table S1, Supporting information; HRESIMS *m/z* 303.1229 $[M + H]^+$ (calcd for $C_{17}H_{19}O_5^+$, 303.1227).

Compound **3**: brown oil; $[\alpha]^{25}_{D}$ -32 (*c* 0.1, MeOH); UV λ_{max} (MeOH) (log ε) 224 (4.53), 245 (sh) (4.15), 286 (4.20), 298 (sh) (4.19) nm; ^{1}H and ^{13}C NMR, see Table 1; HRESIMS *m/z* 557.2537 $[M + H]^+$ (calcd for $C_{34}H_{37}O_7^+$, 557.2534).

Compound **4**: brown oil; $[\alpha]^{25}_{D}$ -47 (*c* 0.1, MeOH); UV λ_{max} (MeOH) (log ε) 224 (4.45), 245 (sh) (4.09), 286 (4.16), 298 (sh) (4.09) nm; ^{1}H and ^{13}C NMR, see Table 1; HRESIMS *m/z* 557.2536 $[M + H]^+$ (calcd for $C_{34}H_{37}O_7^+$, 557.2534).

Compound **5**: brown oil; $[\alpha]^{25}_{D}$ 43 (*c* 0.1, MeOH); UV λ_{max} (MeOH) (log ε) 223 (4.57), 244 (sh) (4.26), 286 (4.32), 298 (sh) (4.28) nm; ^{1}H and ^{13}C NMR, see Table 1; HRESIMS *m/z* 557.2535 $[M + H]^+$ (calcd for $C_{34}H_{37}O_7^+$, 557.2534).

Compound **6**: light yellow oil; $[\alpha]^{25}_{D}$ -26 (*c* 0.1, MeOH); UV λ_{max} (MeOH) (log ε) 224 (4.51), 242 (sh) (4.20), 286 (4.21), 297 (4.21) nm; ^{1}H and ^{13}C NMR, see Table 2; HRESIMS *m/z* 571.2691 $[M + H]^+$ (calcd for $C_{35}H_{39}O_7^+$, 557.2690).

Compound **7**: light yellow plates (toluene-acetone); $[\alpha]^{25}_{D}$ 36 (*c* 0.1, MeOH); UV λ_{max} (MeOH) (log ε) 224 (4.60), 243 (sh) (4.30), 286 (4.31), 296 (4.32) nm; ^{1}H and ^{13}C NMR, see Table 2; HRESIMS *m/z* 571.2691 $[M + H]^+$ (calcd for $C_{35}H_{39}O_7^+$, 557.2690).

Compound **8**: light yellow oil; $[\alpha]^{25}_{D}$ 82 (*c* 0.1, MeOH); UV λ_{max} (MeOH) (log ε) 228 (sh) (4.51), 242 (sh) (4.09), 290 (4.24) nm; ^{1}H and ^{13}C NMR, see Table 2; HRESIMS *m*/*z* 569.2538 $[M + H]^+$ (calcd for $C_{35}H_{37}O_7^+$, 569.2534).

Compound **9**: yellow oil; $[\alpha]^{25}_{D}$ -48 (*c* 0.1, MeOH); UV λ_{max} (MeOH) (log ε) 228 (sh) (4.25), 246 (sh) (3.91), 288 (4.04) nm; ^{1}H and ^{13}C NMR, see Table 3; HRESIMS *m*/*z* 569.2173 $[M + H]^+$ (calcd for $C_{34}H_{33}O_8^+$, 569.2170).

Compound **10**: yellow oil; $[\alpha]^{25}_{D}$ -23 (*c* 0.1, MeOH); UV λ_{max} (MeOH) (log ε) 228 (sh) (4.31), 246 (sh) (4.07), 289 (4.10) nm; ^{1}H and ^{13}C NMR, see Table 3; HRESIMS *m*/*z* 569.2176 $[M + H]^+$ (calcd for $C_{34}H_{33}O_8^+$, 569.2170).

Conformational analysis. Conformational analysis of compound **3** was performed with MacroModel 9.8 software (Schrödinger LLC) employing the OPLS 2005 (Optimized Potential for Liquid Simulations) force field in H_2O.

X-ray Diffraction Analysis. The crystal data and relative configuration of compound **7** was determined using data collected on a STOE StadiVari diffractometer at 123 K using Ga K_α radiation with λ = 1.5418 Å, θ_{max} = 59.5°. The X-Area software was used for data collection and integration. The structure was solved by the charge flipping method using the program Superflip.[48] Plots were produced using MERCURY.[49]

Crystal data of **7** (toluene-acetone): formula $C_{35}H_{38}O_7$, M = 570.68, F(000) = 600, light yellow plates, size 0.03 × 0.09 × 0.13 mm^3, triclinic, space group P1, Z = 2, a = 11.2529(6) Å, b = 11.6760(7) Å, c = 12.6237(9) Å, α = 94.476(6)°, β = 110.367(5)°, γ = 89.929(5)°, V= 1549.58(17)Å^3, D_{calc} = 1.216 Mg m^{-3}. From a total of 21 505 reflections, 9753 were independent (merging r = 0.192). From these, 3179 were considered as observed ($I > 3\ \sigma(I)$) and were used to refine 758 parameters, R = 0.1267 (observed data), wR = 0.3661 (all data), GOF = 1.6334. Minimal/maximal residual electron density = -0.48/0.70 e Å^{-3}.

In Vitro Antifungal Bioassays. Sporangia suspensions of *P. viticola* (2.0-3.0 × 10^5 sporangia/mL) and conidia suspensions of *V. inaequalis* (1.5-2.0 × 10^5 conidia/mL) were prepared by washing fresh, sporulating grapevine leaves or dried, sporulating apple leaves with demineralized water. Sporangia suspensions of *P. infestans* (1.2-1.5 × 10^5 sporangia/mL) were prepared by placing mycelium dispatched from 10-14 days old cultures into demineralized water and shaking vigorously. Suspensions were filtered over a cheese cloth, the concentration was assessed and adjusted using a Thoma cell counting chamber. The protocols to maintain or cultivate the three pathogens were described in detail by Thuerig et al.[11]

For the determination of MIC_{100}, defined as the concentration needed to completely inhibit the activity of zoospores, the crude extract and pure compounds **1-4**, **5-8**, and **10** were dissolved in DMSO at a concentration of 2 mg/mL. Compounds **4** and **9** were not tested due to the insufficient amounts available. Each solution was then serially diluted 1:1 in demineralized water to 3.9 µg/mL. Aliquots of 5 (experiment 1) or 6 µL (experiments 2 and 3) of each concentration were added to 94 µL of the appropriate medium, namely mineral water ("Evian") for *P. viticola*, demineralized water for *V. inaequalis*, and demineralized water containing 1 mL/L V8-medium (200 mL/L Campbell's V8, 3 g/L $CaCO_3$, pH 6.3) for *P. infestans*. Then, 20 µL of the sporangia or conidia suspension was added. Resulting concentrations of the test products were between 0.163 and 83.33 µg/mL (experiment 1) or 0.195 and 100 µg/mL (experiments 2 and 3).

In all in vitro experimental sets, the effect of the solvent (DMSO) alone was tested in at least one replicate in all relevant concentrations.

The activity was assessed 2-3 h (*P. viticola*), one day (*P. infestans*), or two days (*V. inaequalis*) after set-up of the experiment using a binocular at magnifications of 50 to 100-fold. The distinction was made for *P. viticola* between "no zoospores germinated, or all zoospores inactive" and "active zoospores present". For *V. inaequalis* and *P. infestans*, it was between "no germination, or germ tubes ≤0.5 × length of the sporangium/conidium" and "germination". To calculate mean MIC_{100}, data were $\log_2$ transformed. Data were then re-transformed to linear scale.

In Vivo Assays on Seedlings. Plant-pathogen bioassays were carried out under semi-controlled conditions in experimental facilities (greenhouse and growth chambers). Small grapevine (*Vitis vinifera* L.) cv. 'Chasselas' or tomato (*Solanum lycopersicum* L.) cv. 'Marmande' seedlings were transplanted to individual pots (0.275 L) containing a standard substrate ('Einheitserde Typ 0', Gebr. Patzer GmbH & Co. KG) previously amended with 3 g/L of a mineral fertilizer (Tardit 3M, Hauert Günther Düngerwerke GmbH). Plants were grown in the greenhouse at a minimal temperature of 18 °C under natural light. The photoperiod was extended with mercury lamps to 16 hours. Plants were used for bioassays 2 to 3 weeks after transplanting when they had 2-3 (tomato, *P. infestans*) or 3-4 fully developed leaves (grapevine, *P. viticola* and apple, *V. inaequalis*).

Each experimental set included a non-treated non-inoculated control, a water-treated inoculated control, a standard treatment (copper hydroxide, Kocide Opti, DuPont de Nemours) at two concentrations (300 µg/mL and 30 µg/mL of copper). All experiments

included six replicate plants per treatment. A formulation of *I. megistocarpa* extract was used, containing 6% of the extract, 83% of a solvent (ethylacetate) and 11% of an emulsifier (Emulsogen EL 360, Clariant) to enhance the solubility in water. The formulation was added to demineralized water at concentrations of 1 and 0.5 mg plant extract/mL. As a control, a blank formulation was tested at corresponding concentrations.

Plants were sprayed with the test products using an air-assisted hand sprayer (DeVilbiss® Compact MINI HVLP Touch-Up Spray Gun) or an automatic spray cabinet until leaves (adaxial and abaxial side) were completely covered with a dense layer of small droplets. Plants were subsequently left to dry at room temperature before inoculation.

P. viticola inoculum were prepared from previously infected plants by washing freshly sporulating grapevine leaves with water and filtering through cheese cloth. *P. infestans* inoculum was prepared from 10-12 days old cultures. Concentration of the sporangia/conidia suspensions were adjusted to 5×10^4 sporangia/mL (*P. viticola*) or 3 x 10^4 sporangia/mL (*P. infestans*). Plants were spray-incoculated using an air-assisted hand sprayer on the the abaxial (*P. viticola*) or the adaxial (*P. infestans*) leaf side. Inoculated plants were subsequently incubated at 20–21°C and 95–99% of relative humidity (RH) in the light for 24 h. Then, plants were maintained at 20°C, 60–80% RH, and a 16/8-h day/night light regime. For grapevine bioassays, 5 to 6 days after inoculation, plants were incubated over night in the dark at 20°C and 95–99% to promote sporulation. Disease incidence (percentage of leaves with disease symptoms) and disease severity (percentage of leaf area covered by lesions) were assessed 5 days (*P. infestans*) or 6 to 7 days (*P. viticola*) after inoculation. Disease assessments for all pathogens were made using continuous values of percentage based on the EPPO standard scale.[50] Efficacies were calculated according to Abbott[51] as $(1 - (A \times B^{-1})) \times 100$, with A = disease severity on an individual plant and B = mean disease severity of control plants.

ASSOCIATED CONTENT

HPLC-activity profiling of the extract, ^{1}H and ^{13}C NMR spectroscopic data for compounds **1-2**; and 1D and 2D NMR spectra of compounds **3-10**.

AUTHOR INFORMATION

Corresponding Author

*Tel: +41-61-2071534. Fax: +41-61-2071474. E-mail: olivier.potterat@unibas.ch

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Weisenburger, D. D. *Hum. Pathol.* **1993**, *24*, 571-576.
(2) Schwarzenbach, R. P.; Egli, T.; Hofstetter, T. B.; von Gunten, U.; Wehrli, B. *Annu. Rev. Environ. Resour.* **2010**, *35*, 109-136.
(3) Mullin, C. A.; Frazier, M.; Frazier, J. L.; Ashcraft, S.; Simonds, R.; vanEngelsdorp, D.; Pettis, J. S. *PLoS One* **2010**, *5*, e9754.
(4) Bolognesi, C. *Mutat. Res. - Rev. Mutat.* **2003**, *543*, 251-272.
(5) Gilliom, R. J. *Environ. Sci. Technol.* **2007**, *41*, 3408-3414.
(6) Dang, Q. L.; Lim, C. H.; Kim, J.-C. *Res. Plant Dis.* **2012**, *18*, 175-185.
(7) Barkai-Golan, R., Chapter 9: Chemical Control. In *Postharvest Diseases of Fruits and Vegetables*, Elsevier: Amsterdam, 2001; pp 147-188.
(8) Ntalli, N. G.; Menkissoglu-Spiroudi, U., Chapter 1: Pesticides of Botanical Origin: A Promising Tool in Plant Protection. In *Pesticides - Formulations, Effects, Fate*, Stoytcheva, M., Ed. InTech: Rijeka, 2011; pp 3-24.
(9) Schnee, S.; Queiroz, E. F.; Voinesco, F.; Marcourt, L.; Dubuis, P.-H.; Wolfender, J.-L.; Gindro, K. *J. Agric. Food Chem.* **2013**, *61*, 5459-5467.
(10) Castillo, F.; Hernandez, D.; Gallegos, G.; Rodriguez, R.; Aguilar, C. N., Chapter 4: Antifungal Properties of Bioactive Compounds from Plants. In *Fungicides for Plant and Animal Diseases*, Dhanasekaran, D.; Thajuddin, N.; Panneerselvam, A., Eds. InTech: Rijeka, 2011; pp 91-106.
(11) Thuerig, B.; Ramseyer, J.; Hamburger, M.; Oberhänsli, T.; Potterat, O.; Schärer, H.-J.; Tamm, L. *Pest Manag. Sci.* **2016**, *72*, 1718-1726.
(12) Potterat, O.; Hamburger, M. *Planta Med.* **2014**, *80*, 1171-1181.
(13) Jaramillo, T. S., *48. Myristicaceae*. Flora of Ecuador, Botanical Institute, Göteborg University: Göteborg, 2004; Vol. 72.
(14) Mabberley, D. J., *Mabberley's Plant-book: A Portable Dictionary of Plants, Their Classification and Uses*. Third ed.; Cambridge University Press: Cambridge, 2008.
(15) *Iryanthera* spp. http://tropicos.org (accessed 06.11.17).
(16) Gentry, A. H. *Ann. Mo. Bot. Gard.* **1975**, *62*, 474-479.
(17) *Iryanthera megistocarpa*. http://tropicos.org (accessed 07.11.17).
(18) Kawanishi, K.; Takagaki, T.; Hashimoto, Y. *Phytochemistry* **1990**, *29*, 2735-2736.
(19) Villamil Z., E.; Cuca S., L. E.; Martinez V., J. C. *Spectroscopy* **1988**, *6*, 157-165.
(20) Bernal, F. A.; Cuca Suarez, L. E. *Biochem. Syst. Ecol.* **2009**, *37*, 772-775.
(21) Silva, D. H. S.; Davino, S. C.; Barros, S. B. d. M.; Yoshida, M. *J. Nat. Prod.* **1999**, *62*, 1475-1478.

(22) Mesa-Siverio, D.; Machin, R. P.; Estevez-Braun, A.; Ravelo, A. G.; Lock, O. *Biorg. Med. Chem.* **2008**, *16*, 3387-3394.
(23) Aponte, J. C.; Vaisberg, A. J.; Rojas, R.; Caviedes, L.; Lewis, W. H.; Lamas, G.; Sarasara, C.; Gilman, R. H.; Hammond, G. B. *J. Nat. Prod.* **2008**, *71*, 102-105.
(24) Martinez V., J. C. *Phytochemistry* **2000**, *55*, 505-511.
(25) Martinez V., J. C.; Cuca S., L. E., Chemical Studies of Myristicaceae Species of the Colombian Amazon. In *Chemistry of the Amazon*, American Chemical Society: Washington DC, 1995; pp 116-123.
(26) Silva, D. H. S.; Cavalheiro, A. J.; Yoshida, M.; Gottlieb, O. R. *Phytochemistry* **1995**, *38*, 1013-1016.
(27) Martinez, V.; Juan, C.; Cuca, S.; Luis, E. *Rev. Colomb. Quim.* **1989**, *18*, 37-46.
(28) Conserva, L. M.; Yoshida, M.; Gottlieb, O. R.; Martinez V., J. C.; Gottlieb, H. E. *Phytochemistry* **1990**, *29*, 3911-3918.
(29) Diaz D, P. P.; De Diaz, A. M. P. *Phytochemistry* **1986**, *25*, 2395-2397.
(30) Vieira, P. C.; Gottlieb, O. R.; Gottlieb, H. E. *Phytochemistry* **1983**, *22*, 2281-2286.
(31) Braz Filho, R.; Da Silva, M. S.; Gottlieb, O. R. *Phytochemistry* **1980**, *19*, 1195-1197.
(32) Garzon N., L.; Cuca S., L. E.; Martinez V., J. C.; Yoshida, M.; Gottlieb, O. R. *Phytochemistry* **1987**, *26*, 2835-2837.
(33) Lopes, N. P.; Silva, D. H. S.; Kato, M. J.; Yoshida, M. *Phytochemistry* **1998**, *49*, 1405-1410.
(34) Ming, D. S.; Lopez, A.; Hillhouse, B. J.; French, C. J.; Hudson, J. B.; Towers, G. H. N. *J. Nat. Prod.* **2002**, *65*, 1412-1416.
(35) Motter Magri, F. M.; Kato, M. J.; Yoshida, M. *Phytochemistry* **1996**, *43*, 669-671.
(36) Silva, D. H. S.; Yoshida, M.; Kato, M. J. *Phytochemistry* **1997**, *46*, 579-582.
(37) Conserva, L. M.; Yoshida, M.; Gottlieb, O. R. *Phytochemistry* **1990**, *29*, 3986-3988.
(38) Bernal, F. A.; Cuca-Suarez, L. E.; Yamaguchi, L. F.; Coy-Barrera, E. D. *Records of Natural Products* **2013**, *7*, 152.
(39) Vieira, P. C.; Yoshida, M.; Gottlieb, O. R.; Paulino Filho, H. F.; Nagem, T. J.; Braz Filho, R. *Phytochemistry* **1983**, *22*, 711-713.
(40) Silva, D. H. S.; Pereira, F. C.; Zanoni, M. V. B.; Yoshida, M. *Phytochemistry* **2001**, *57*, 437-442.
(41) Silva, D. H. S.; Zhang, Y.; Santos, L. A.; Bolzani, V. S.; Nair, M. G. *J. Agric. Food Chem.* **2007**, *55*, 2569-2574.
(42) Morais, S. K. R.; Teixeira, A. F.; dos S. Torres, Z. E.; Nunomura, S. M.; Yamashiro-Kanashiro, E. H.; Lindoso, J. A. L.; Yoshida, M. *J. Braz. Chem. Soc.* **2009**, *20*, 1110-1118.
(43) Braz Filho, R.; Diaz D, P. P.; Gottlieb, O. R. *Phytochemistry* **1980**, *19*, 455-459.
(44) de Almeida, M. E. L.; Braz Filho, R.; von Bülow, M. V.; Correa, J. J. L.; Gottlieb, O. R.; Maia, J. G. S.; da Silva, M. S. *Phytochemistry* **1979**, *18*, 1015-1016.
(45) de Lima, R. A.; Franca, N. C.; Diaz Diaz, P.; Gottlieb, O. R. *Phytochemistry* **1975**, *14*, 1831-1833.
(46) Gottlieb, O. R. *J. Ethnopharmacol.* **1979**, *1*, 309-323.
(47) Lopez, A.; Hudson, J. B.; Towers, G. H. N. *J. Ethnopharmacol.* **2001**, *77*, 189-196.
(48) Palatinus, L.; Chapuis, G. *J. Appl. Crystallogr.* **2007**, *40*, 786-790.
(49) Macrae, C. F.; Bruno, I. J.; Chisholm, J. A.; Edgington, P. R.; McCabe, P.; Pidcock, E.; Rodriguez-Monge, L.; Taylor, R.; van de Streek, J.; Wood, P. A. *J. Appl. Crystallogr.* **2008**, *41*, 466-470.
(50) *EPPO Bulletin* **2001**, *31*, 313-317.
(51) Abbott, W. S. *J. Econ. Entomol.* **1925**, *18*, 265-267.

SUPPORTING INFORMATION

Flavonolignans from *Iryanthera megistocarpa* with Inhibitory Activity against Major Agricultural Pathogens

Justine Ramseyer,[†] Barbara Thuerig,[‡] Maria De Mieri,[†] Mengjie Zhu,[†] Markus Neuburger,[§] Hans-Jakob Schärer,[‡] Thomas Oberhänsli,[‡] Mahabir P. Gupta,[⊥] Lucius Tamm,[‡] Matthias Hamburger,[†] and Olivier Potterat*[,†]

[†]Division of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel, Klingelbergstrasse 50, 4056 Basel, Switzerland

[‡]Research Institute of Organic Agriculture FiBL, Department of Crop Sciences, Ackerstrasse 113, 5070 Frick, Switzerland

[§]Department of Chemistry, University of Basel, Mattenstrasse 24a, 4058 Basel, Switzerland

[⊥]Center for Pharmacognostic Research on Panamanian Flora, Faculty of Pharmacy, University of Panama, Panama City, Republic of Panama

Corresponding Author

*Tel: +41-61-2071534. Fax: +41-61-2071474. E-mail: olivier.potterat@unibas.ch

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Table S1. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds **1** and **2** (DMSO-d_6; 500 MHz for ^{1}H, ^{13}C extracted from ^{1}H-^{13}C 2D inverse detected experiments; δ in ppm)

	1			**2**	
position	δ_H, (J in Hz)	δ_C, type	position	δ_H, (J in Hz)	δ_C, type
1	-	134.9, C	1	-	132.9, C
2	6.79, br s	108.3, CH	2, 6	7.13, d (8.5, 2.0)	128.6, CH
3	-	146.8, C	3, 5	6.83 d (8.5, 2.0)	113.5, C
4	-	144.9, C	4	-	157.0, C
5	6.77, d (8.0)	107.7, CH			
6	6.67, d (8.0)	120.6, CH			
7	2.80, t (7.5)	29.6, CH_2	7	2.82, t (7.5)	29.0, CH_2
8	3.18, t (7.5)	44.9, CH_2	8	3.18, t (7.5)	44.9, CH_2
9	-	203.3, C	9	-	203.3, C
10	-	104.0, C	10	-	103.9, C
11	-	165.3, C	11	-	165.0, C
12	5.89, d (1.5)	95.5, CH	12	5.91, d (2.1)	95.3, CH
13	-	164.7, C	13	-	164.5, C
14	5.97, d (4.5)	91.1, CH	14	5.99, d (2.1)	91.1, CH
15	-	162.6, C	15	-	162.6, C
16	3.79, s	55.4, CH_3	16	3.80, s	55.3, CH_3
17	5.93, s	100.1, CH_2	17	3.71, s	54.5, CH_3

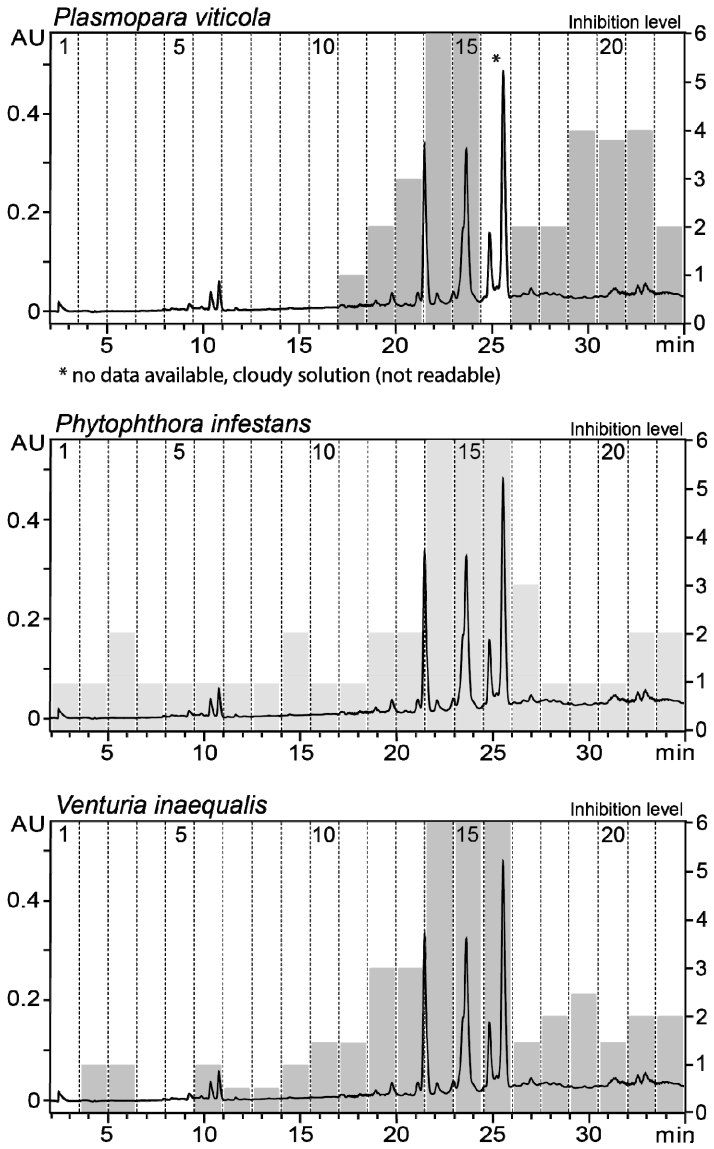

Figure S1. HPLC-profiling of *Iryanthera megistocarpa* ethyl acetate extract against *Plasmopara viticola* (**1**), *Venturia inaequalis* (**2**), and *Phytophthora infestans* (**3**).

Separations were performed on a SunFire C_{18} (5 µm, 150 x 10 mm i.d.). Mobile phase consisted of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B); gradient of 5 to 100% B in 30 min, followed by 100% for 5 min; flow rate of 4.0 mL/min; detection at 254 nm. Collection every 90 sec from 2 to 35 min (22 fractions). 20 mg of extract injected in 2 portions. After drying, micro-fractions were re-dissolved in 100 µL DMSO. 6 µL of each fraction was added to 96-well plates containing 94 µL of the appropriate medium for each pathogen. The fractions were then serially diluted in the test plate 1:10 and 1:100 with the corresponding medium. Next, 20 µL of a continuously stirred sporangia or conidia suspension were added to each well. Inhibition levels were then scored as follows: 0 = similar to water control; 1 = distinct reduction in number and/or activity of zoospores (*P. viticola*), or distinct reduction in germination rate and/or length of germ tubes (*P. infestans* and *V. inaequalis*); 2 = no zoospores germinated, or all zoospores inactive (*P. viticola*), or no germination, or germ tubes ≤0.5 × length of the sporangium/conidium (*P. infestans and V. inaequalis*). Inhibition levels at the three concentrations were summed up, resulting in values between 0 (no inhibition at highest tested concentration) and 4 (complete inhibition down to lowest tested concentration).

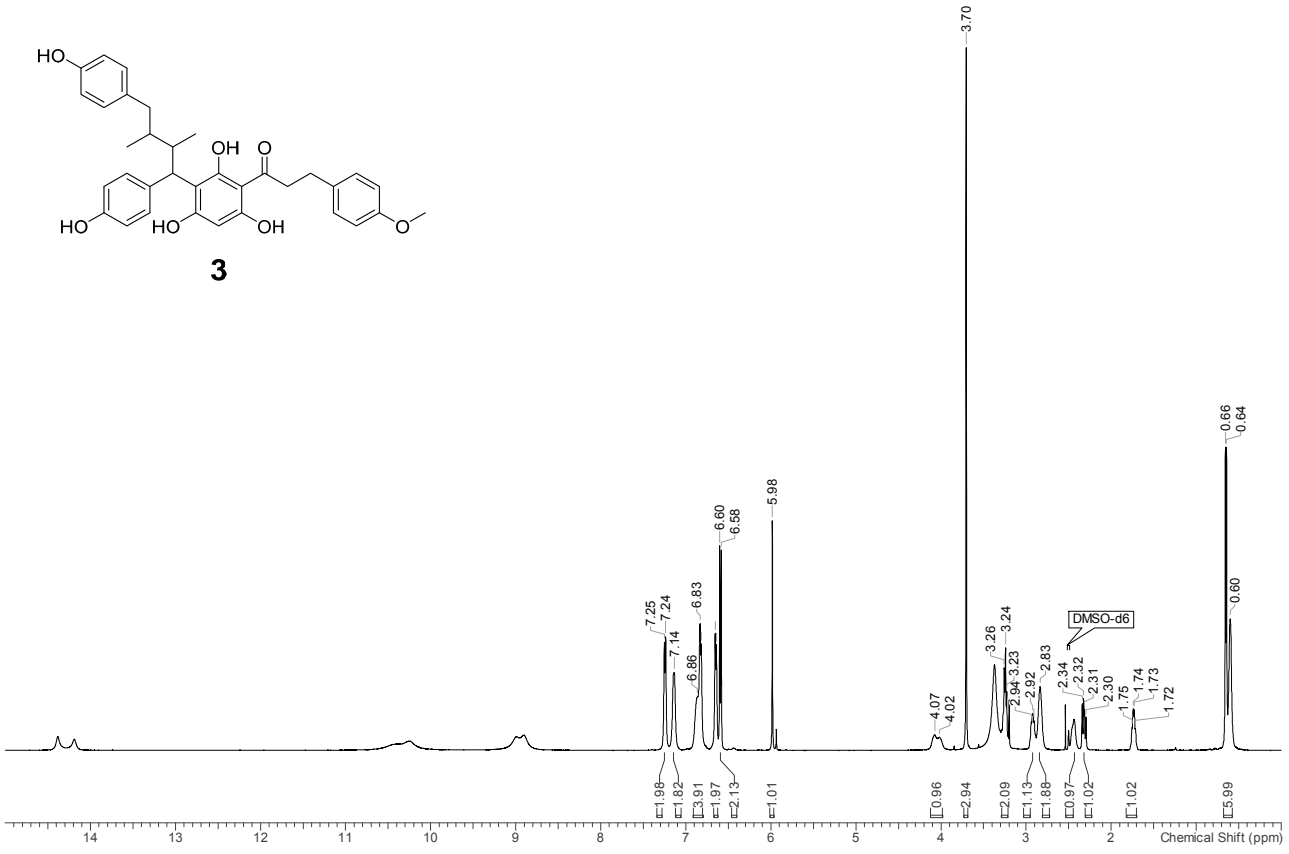

Figure S2. [1]H NMR spectrum of compound **3** (500 MHz, DMSO-d_6)

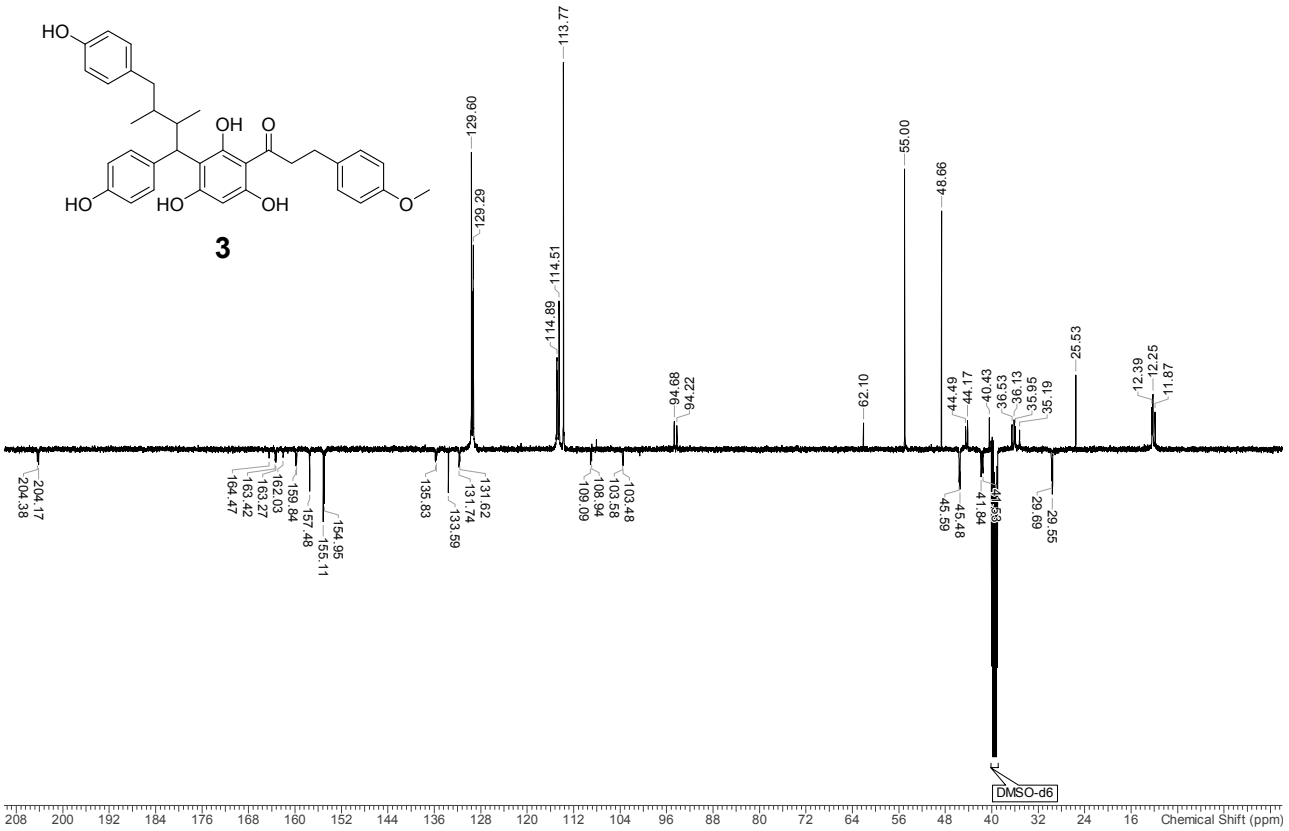

Figure S3. [13]C-DEPTq spectrum of compound **3** (125 MHz, DMSO-d_6)

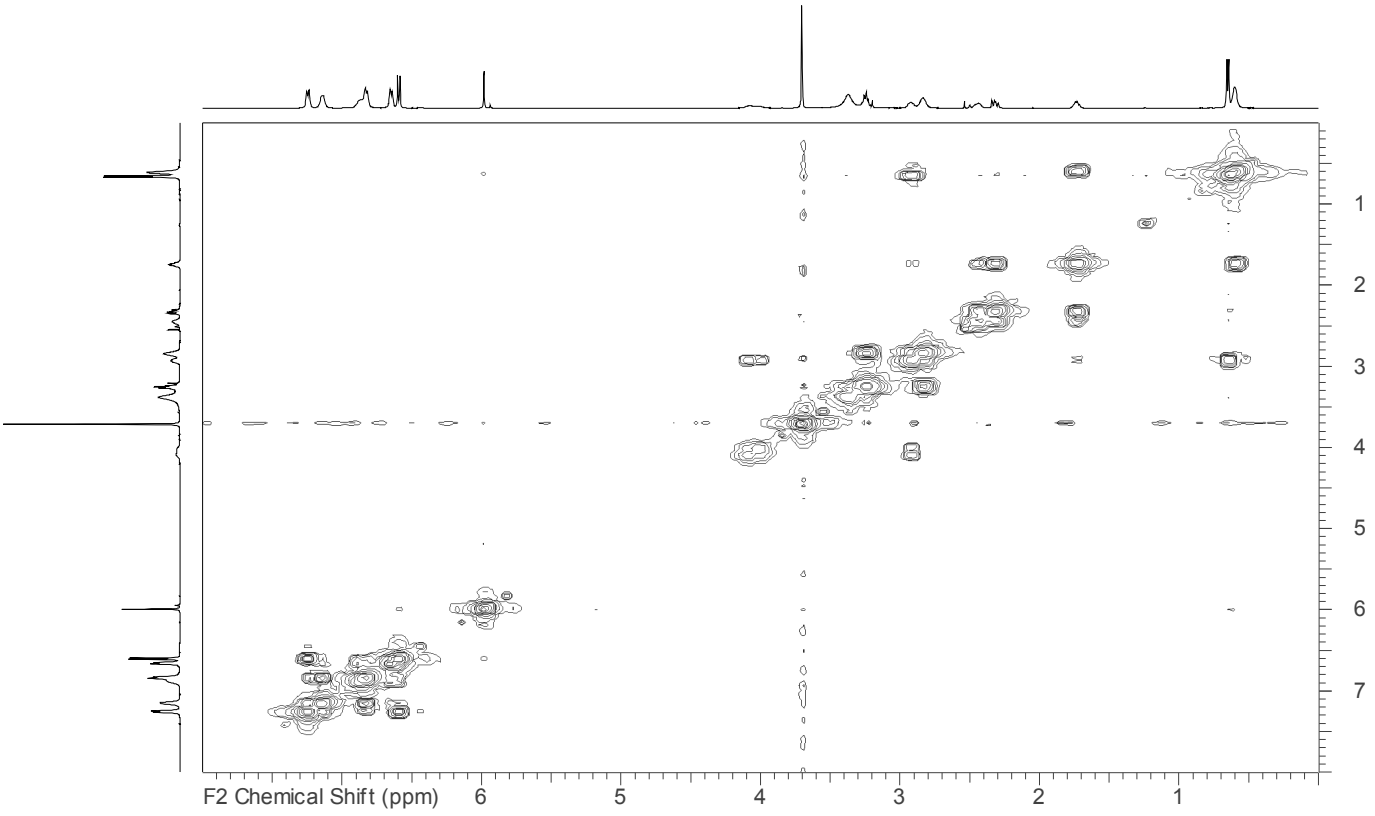

Figure S4. ^{1}H-^{1}H COSY spectrum of compound **3** (500 MHz, DMSO-d_6)

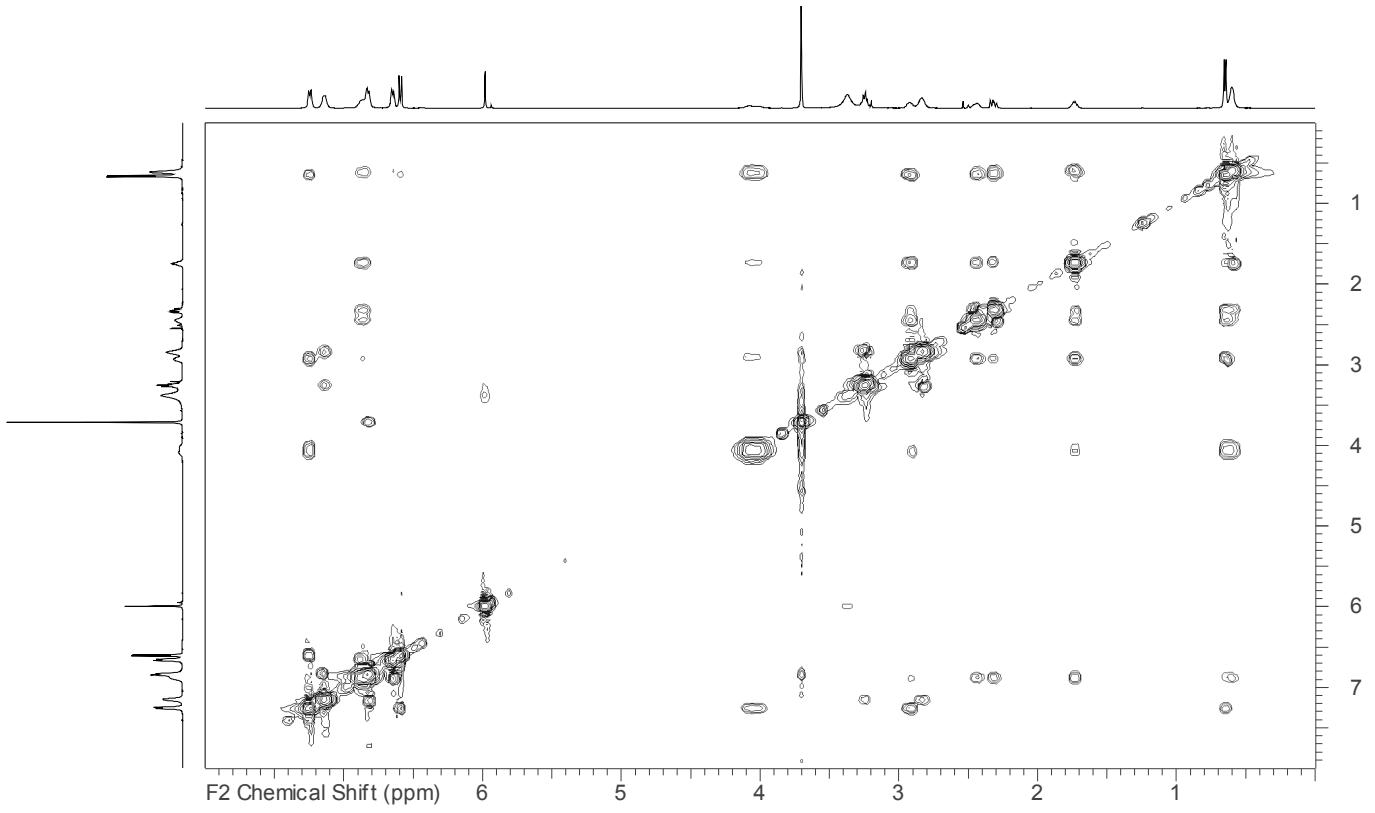

Figure S5. ^{1}H-^{1}H ROESY spectrum of compound **3** (500 MHz, DMSO-d_6)

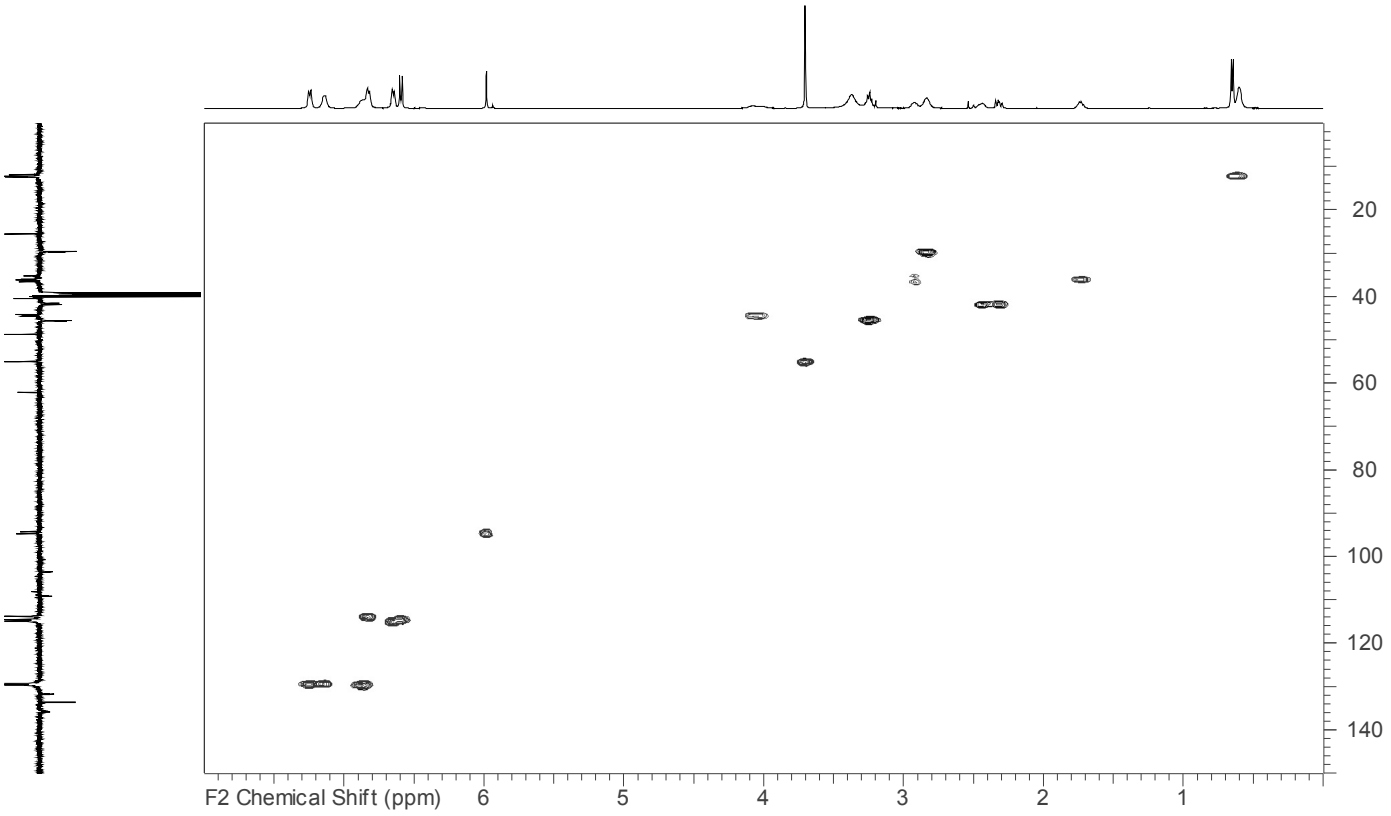

Figure S6. HSQC-DEPT spectrum of compound **3** (500 MHz, DMSO-d_6)

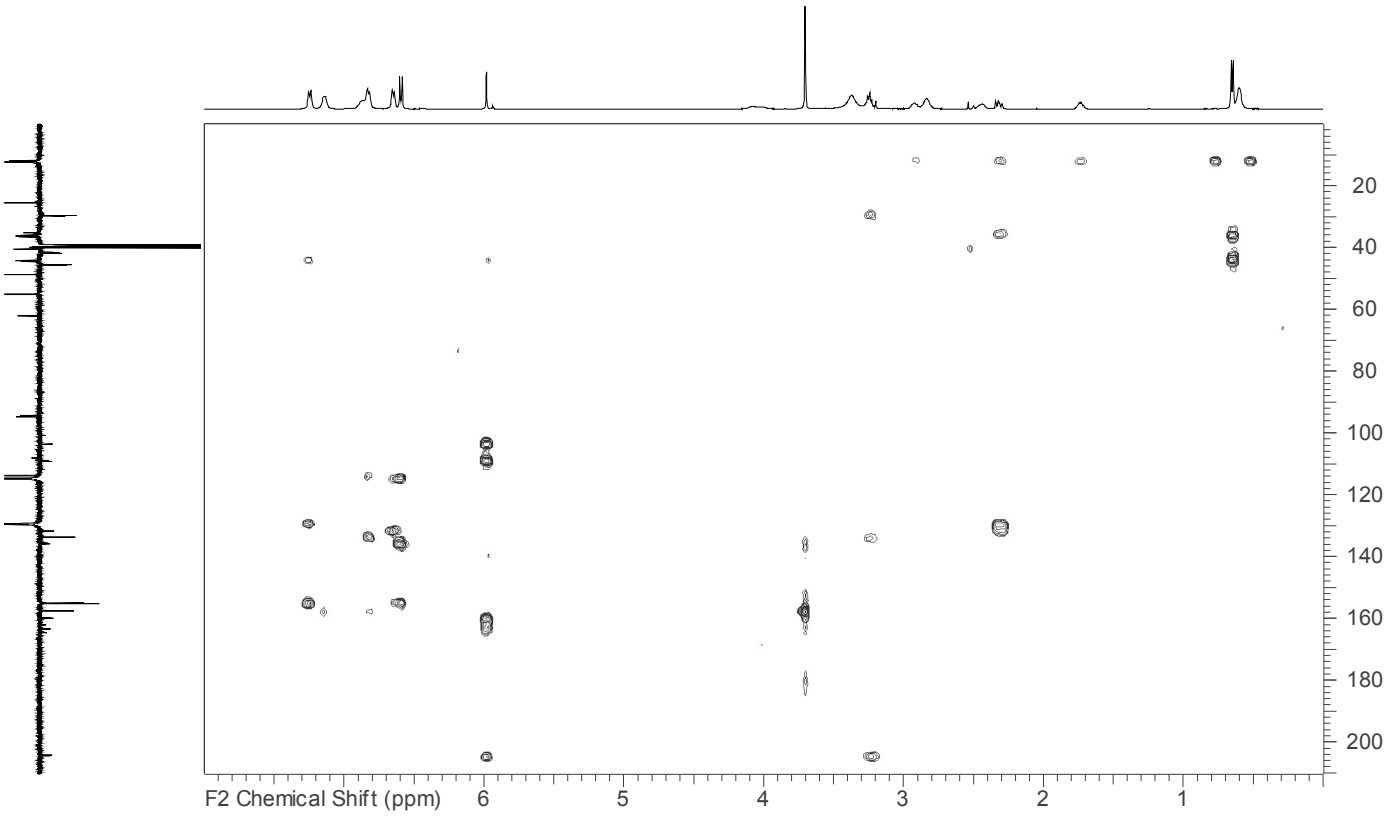

Figure S7. HMBC spectrum of compound **3** (500 MHz, DMSO-d_6)

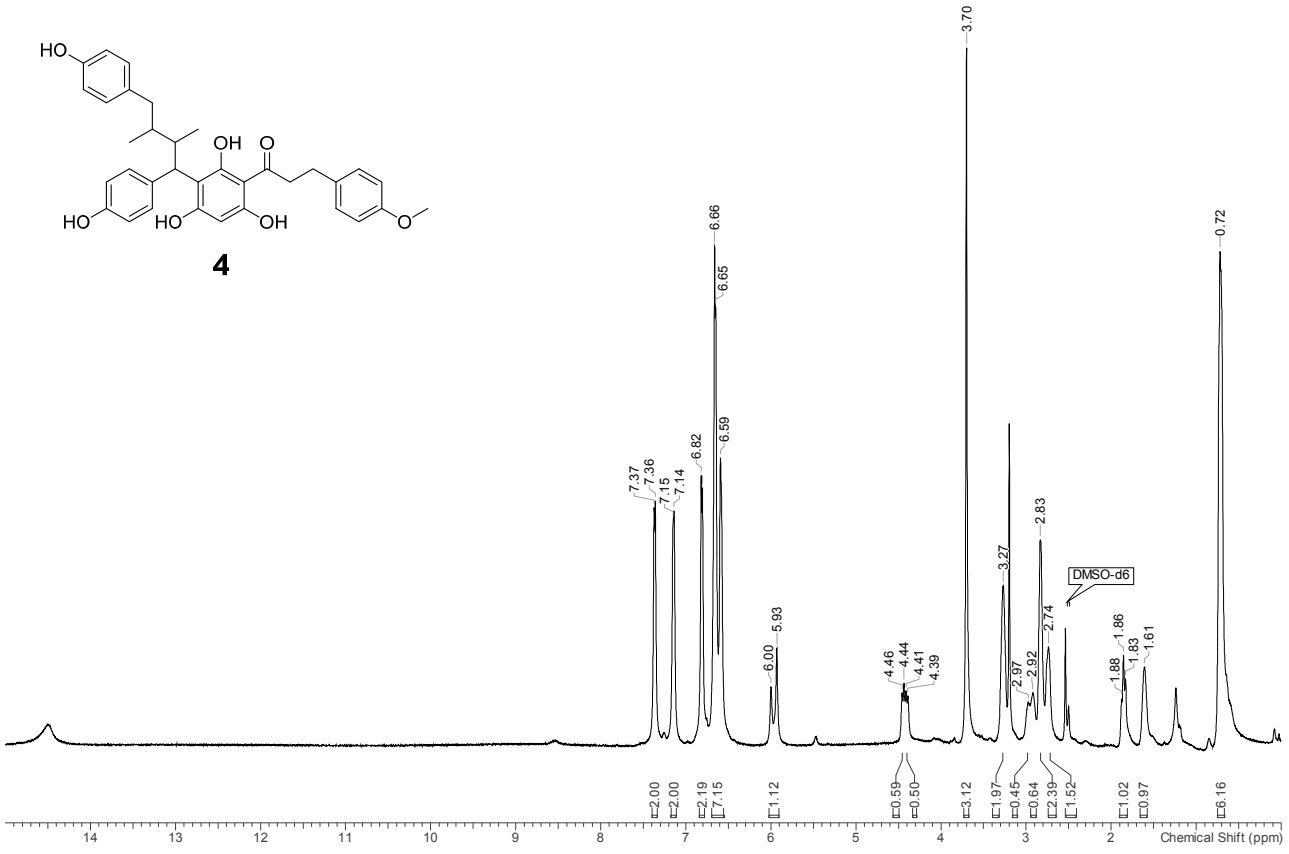

Figure S8. ^{1}H NMR spectrum of compound **4** (500 MHz, DMSO-d_6)

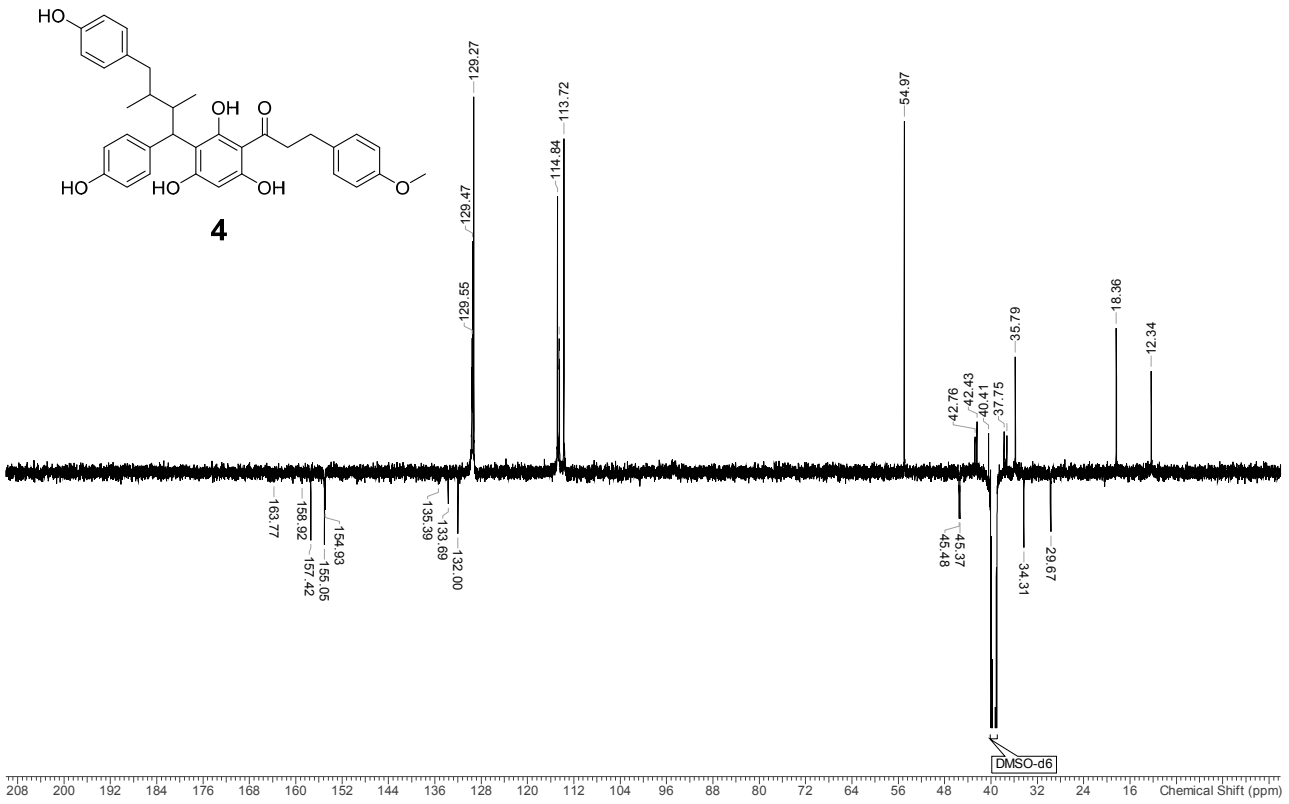

Figure S9. ^{13}C-DEPTq spectrum of compound **4** (125 MHz, DMSO-d_6)

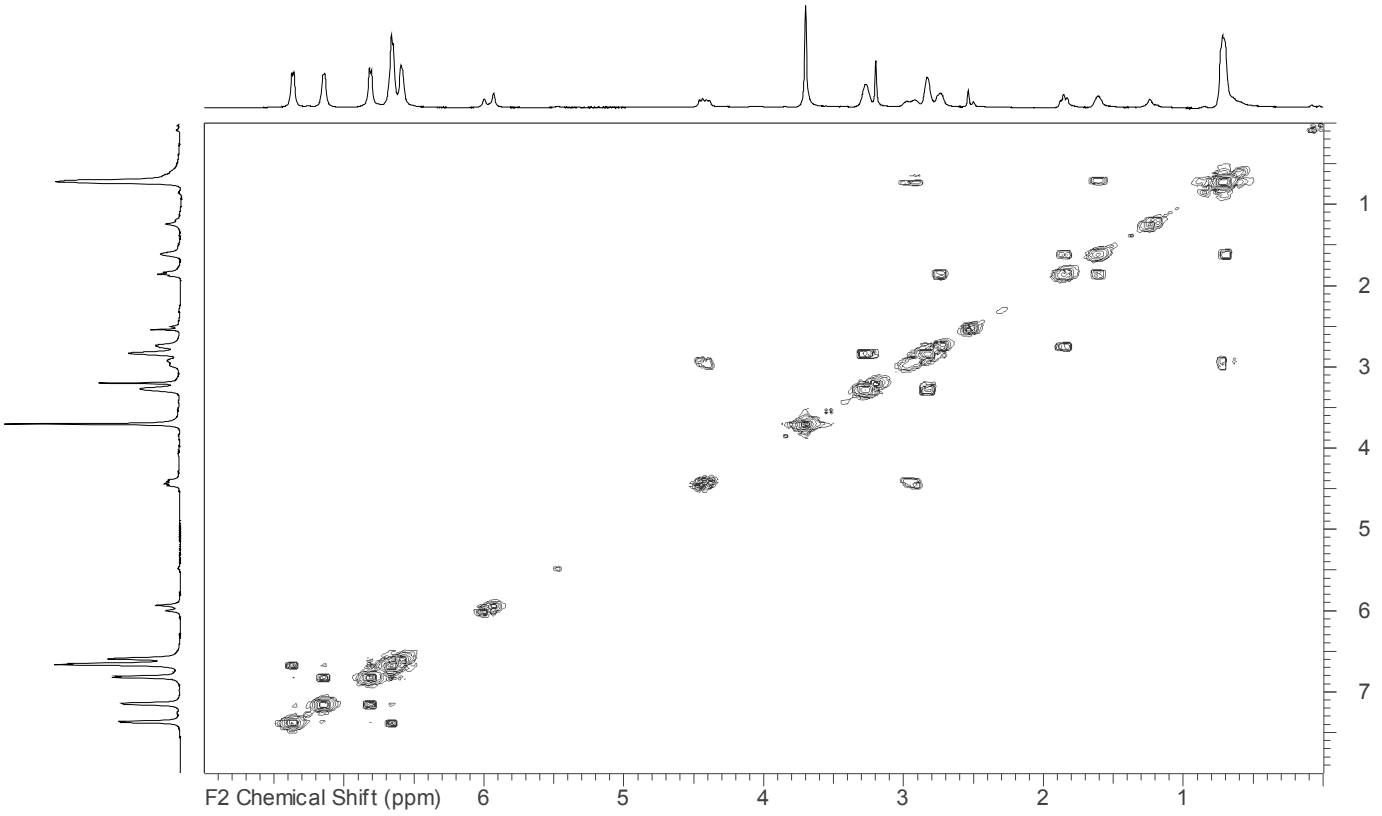

Figure S10. ^{1}H-^{1}H COSY spectrum of compound **4** (500 MHz, DMSO-d_6)

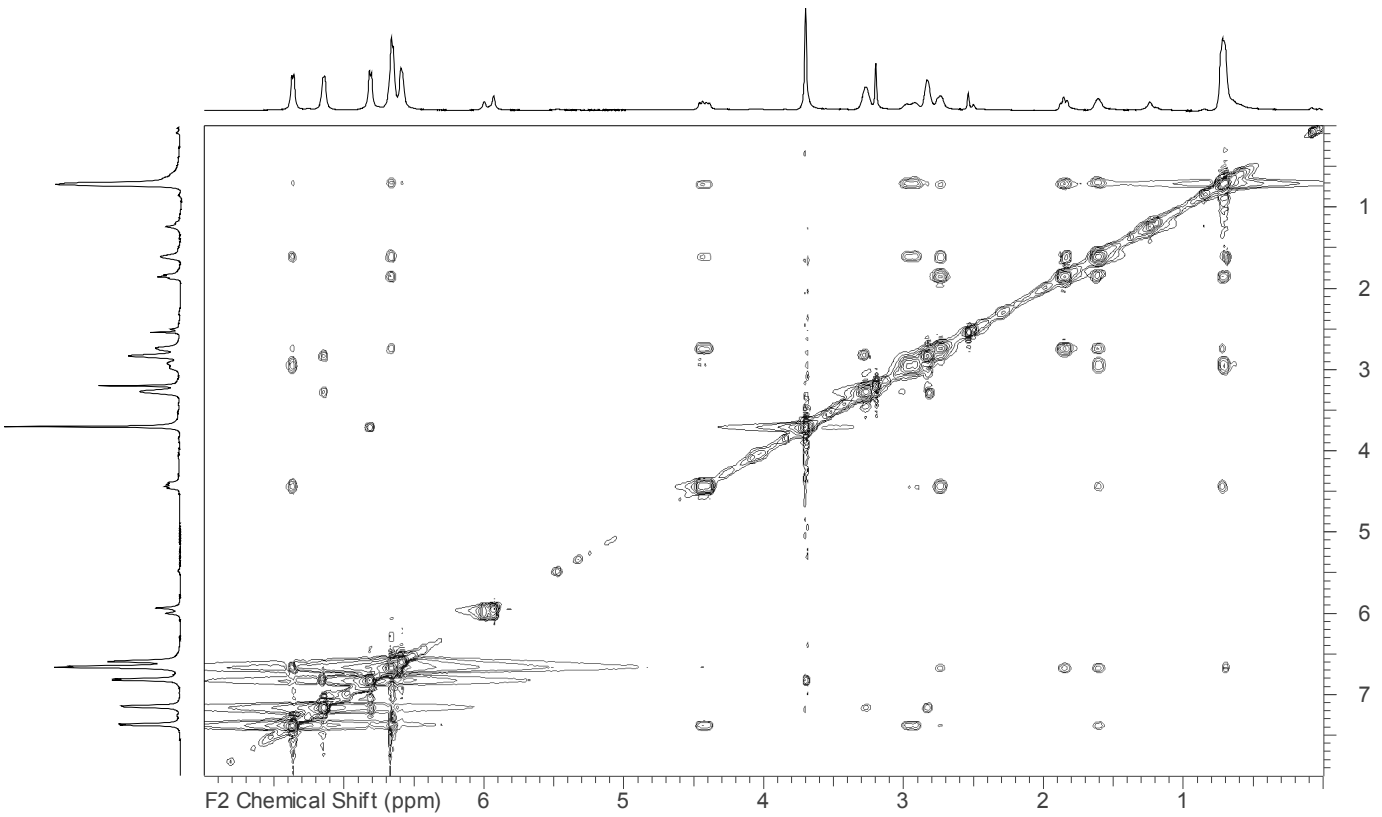

Figure S11. ^{1}H-^{1}H ROESY spectrum of compound **4** (500 MHz, DMSO-d_6)

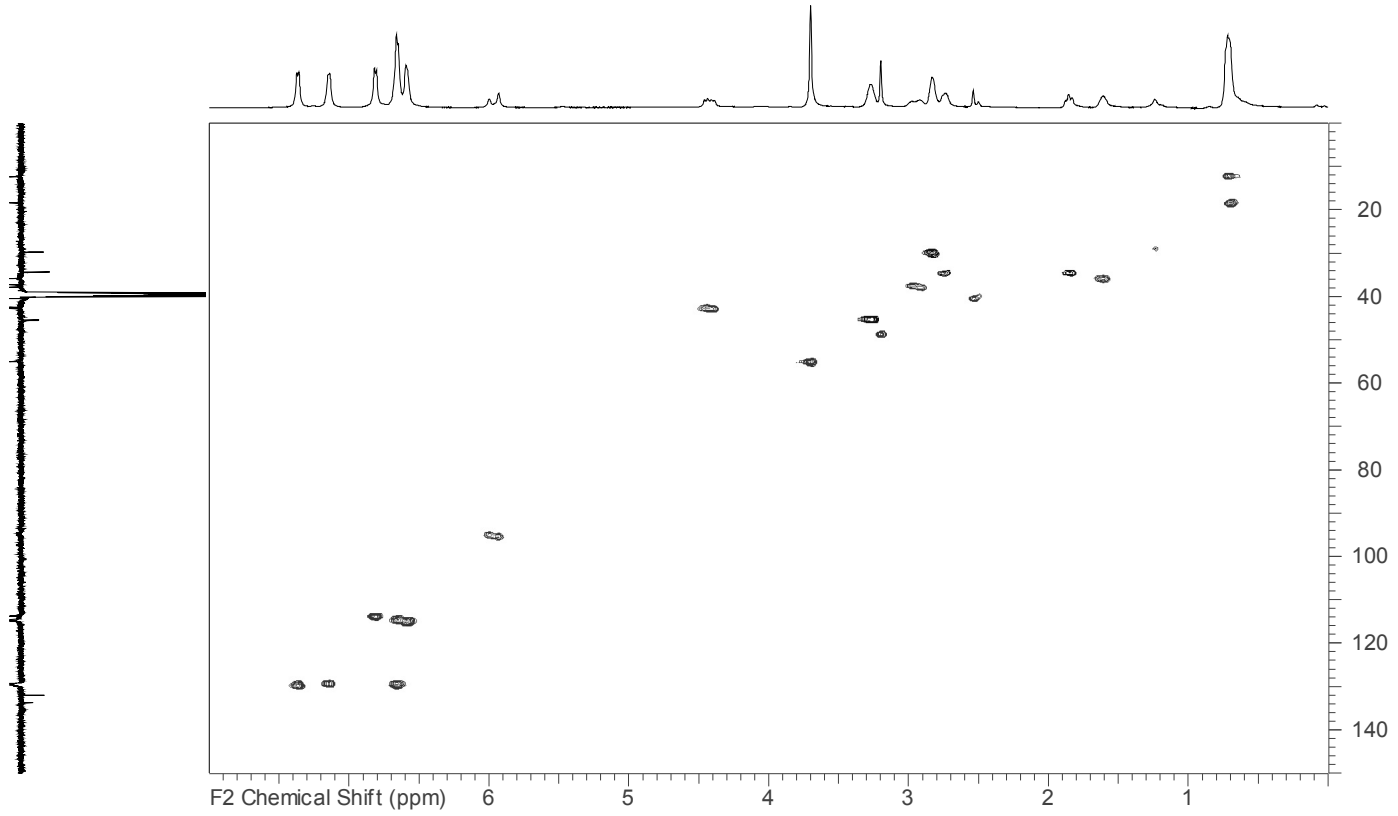

Figure S12. HSQC-DEPT spectrum of compound **4** (500 MHz, DMSO-d_6)

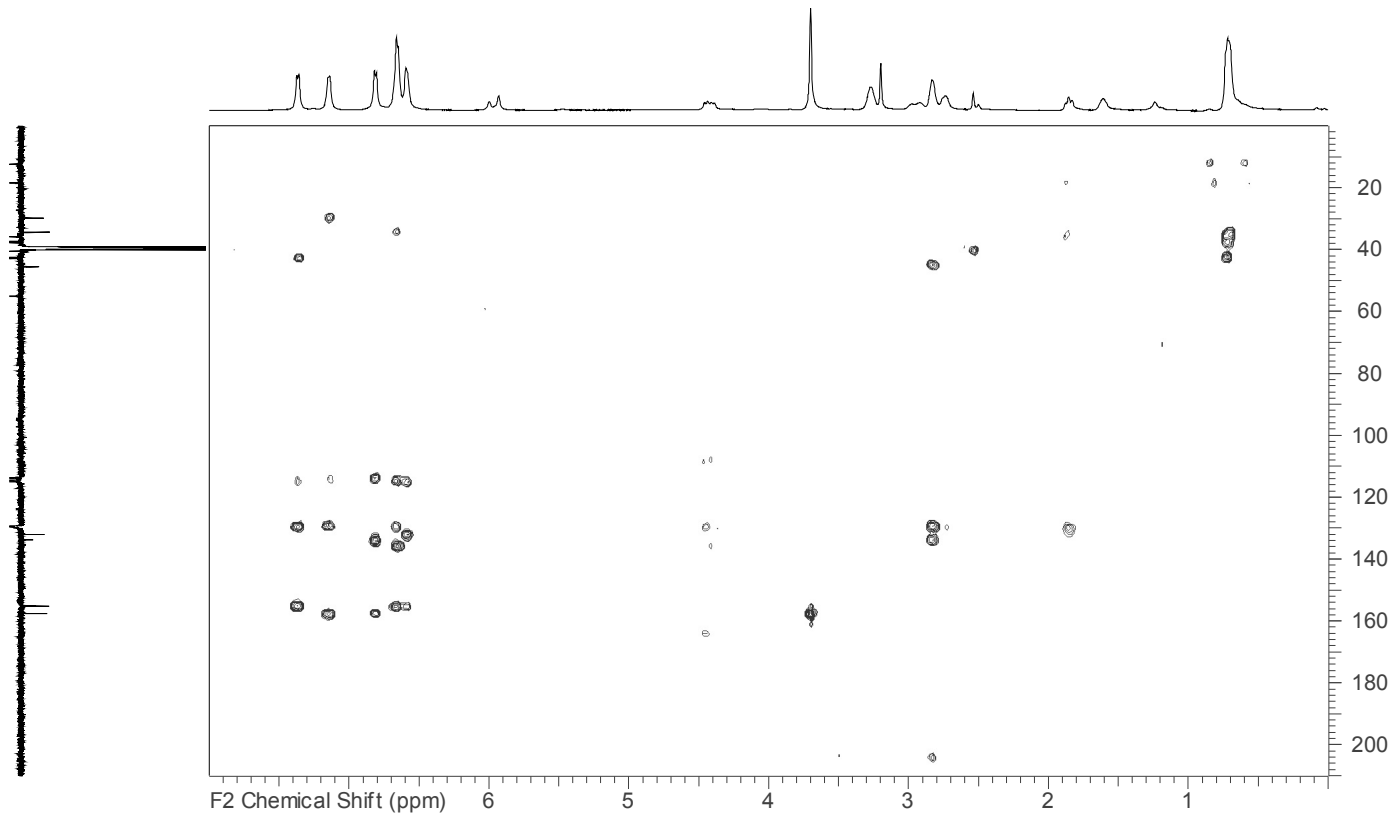

Figure S13. HMBC spectrum of compound **4** (500 MHz, DMSO-d_6)

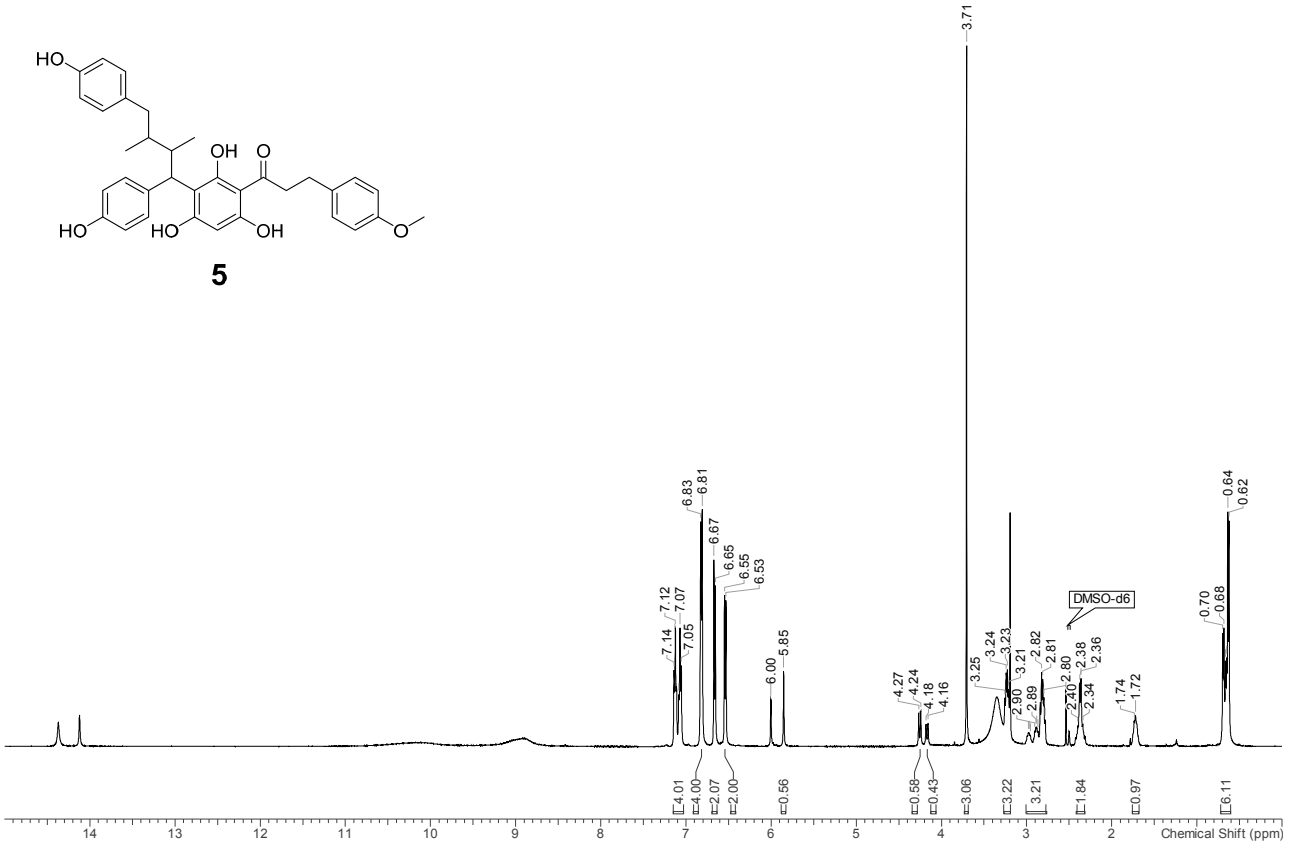

Figure S14. ^{1}H NMR spectrum of compound **5** (500 MHz, DMSO-d_6)

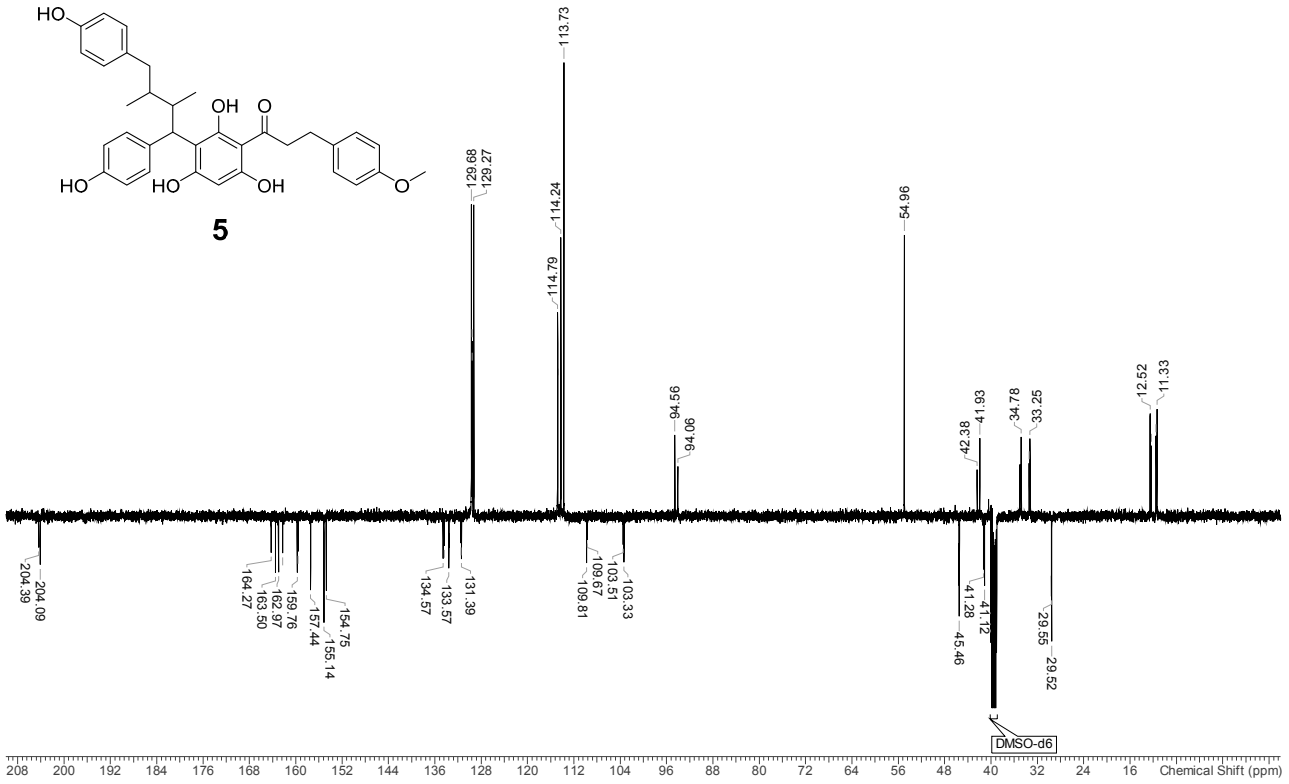

Figure S15. ^{13}C-DEPTq spectrum of compound **5** (125 MHz, DMSO-d_6)

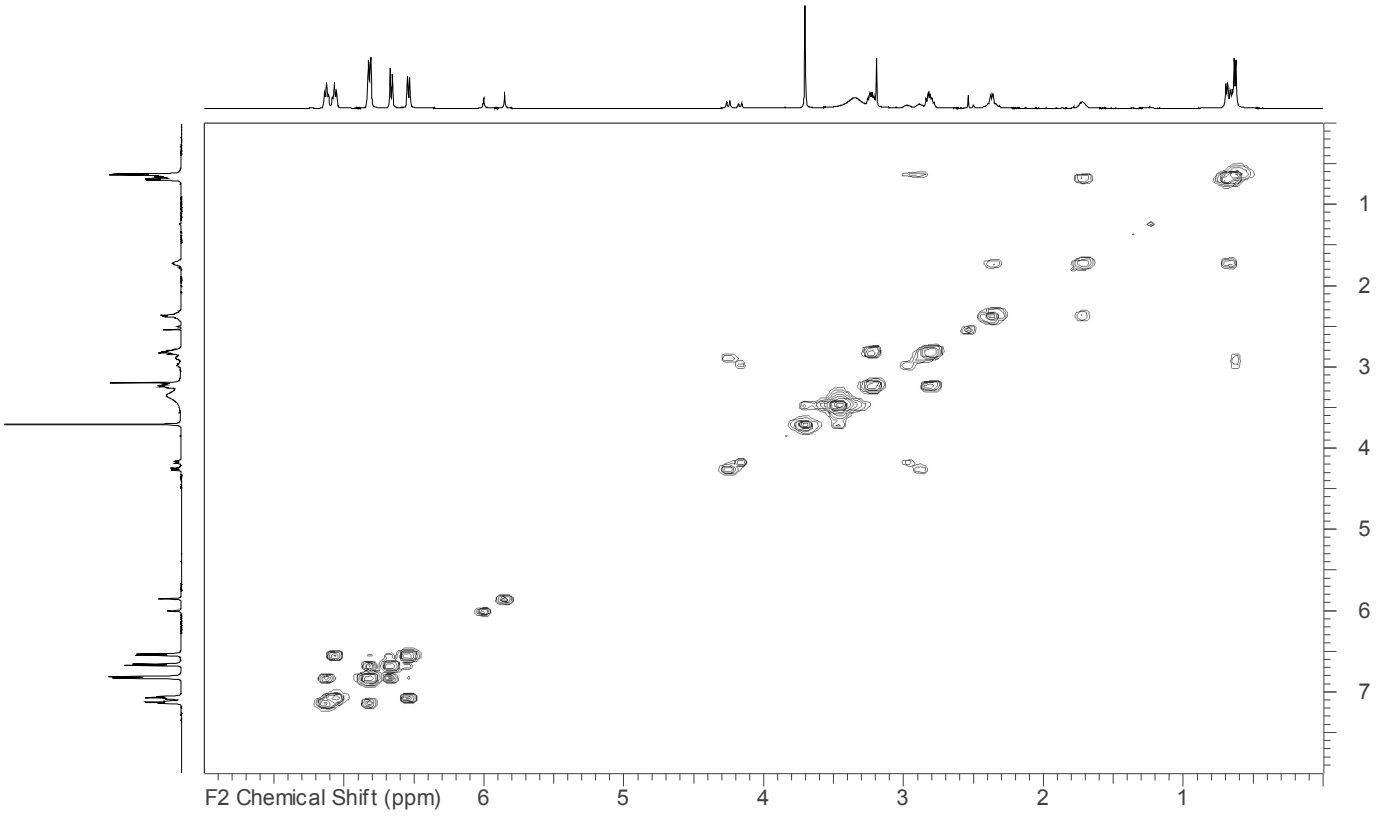

Figure S16. ^{1}H-^{1}H COSY spectrum of compound **5** (500 MHz, DMSO-d_6)

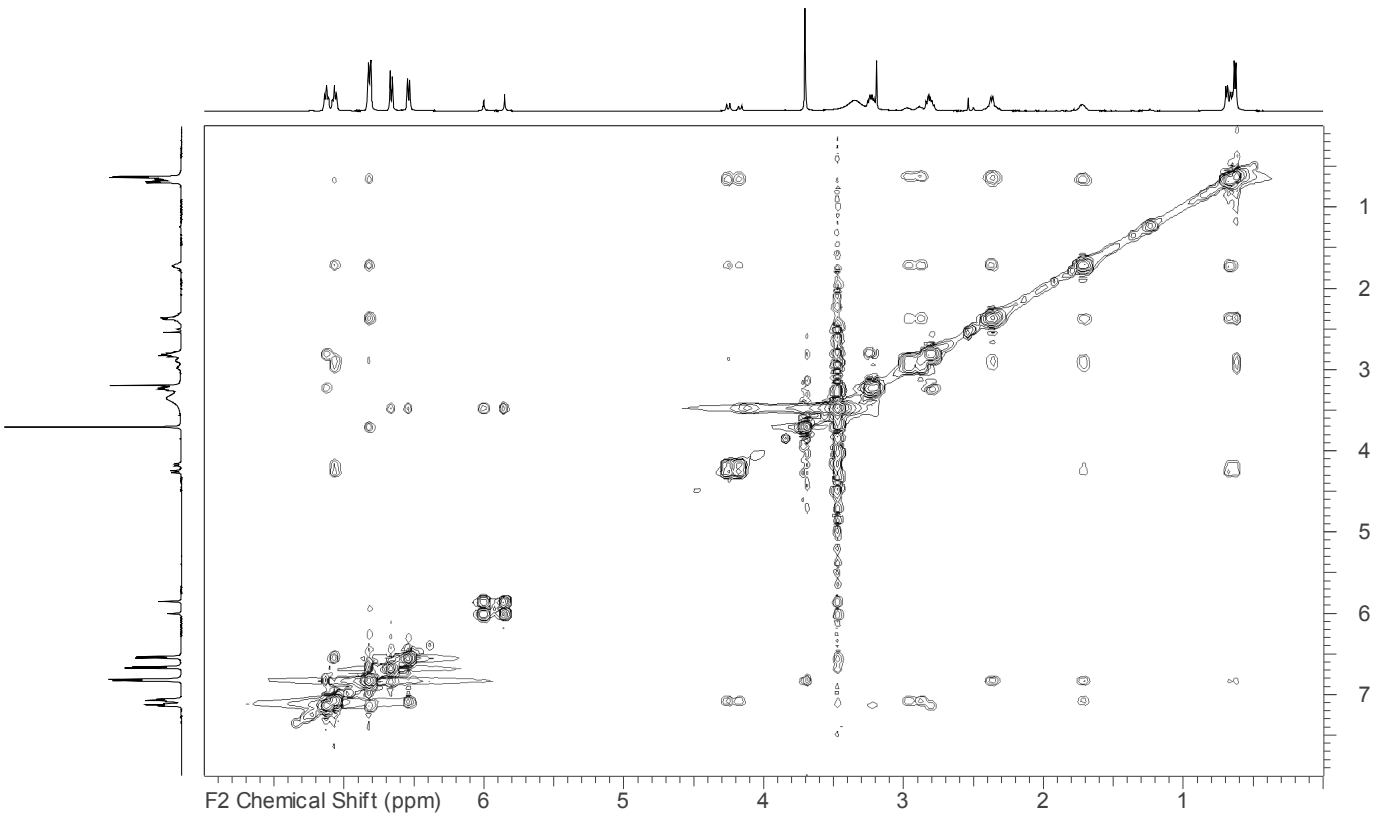

Figure S17. ^{1}H-^{1}H ROESY spectrum of compound **5** (500 MHz, DMSO-d_6)

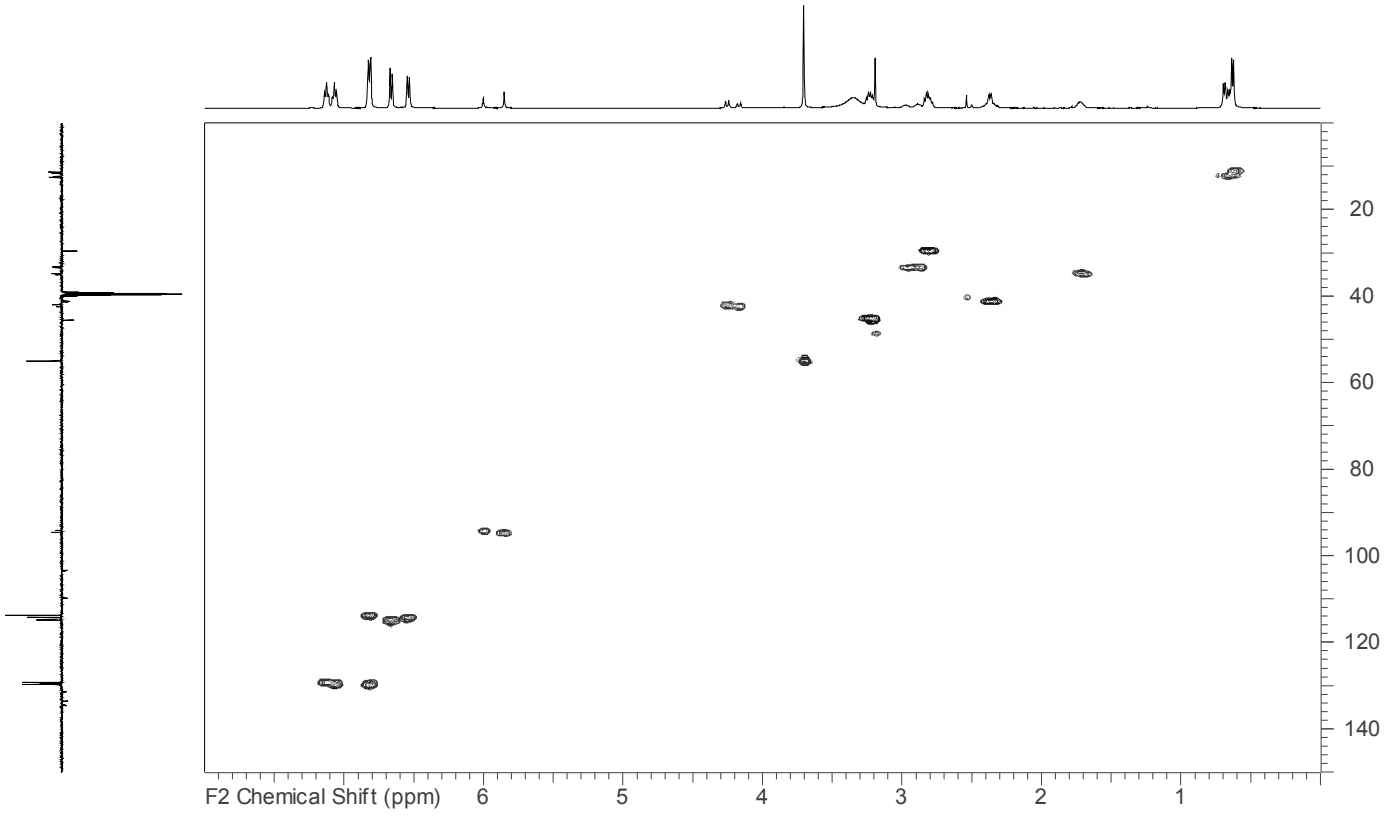

Figure S18. HSQC-DEPT spectrum of compound **5** (500 MHz, DMSO-d_6)

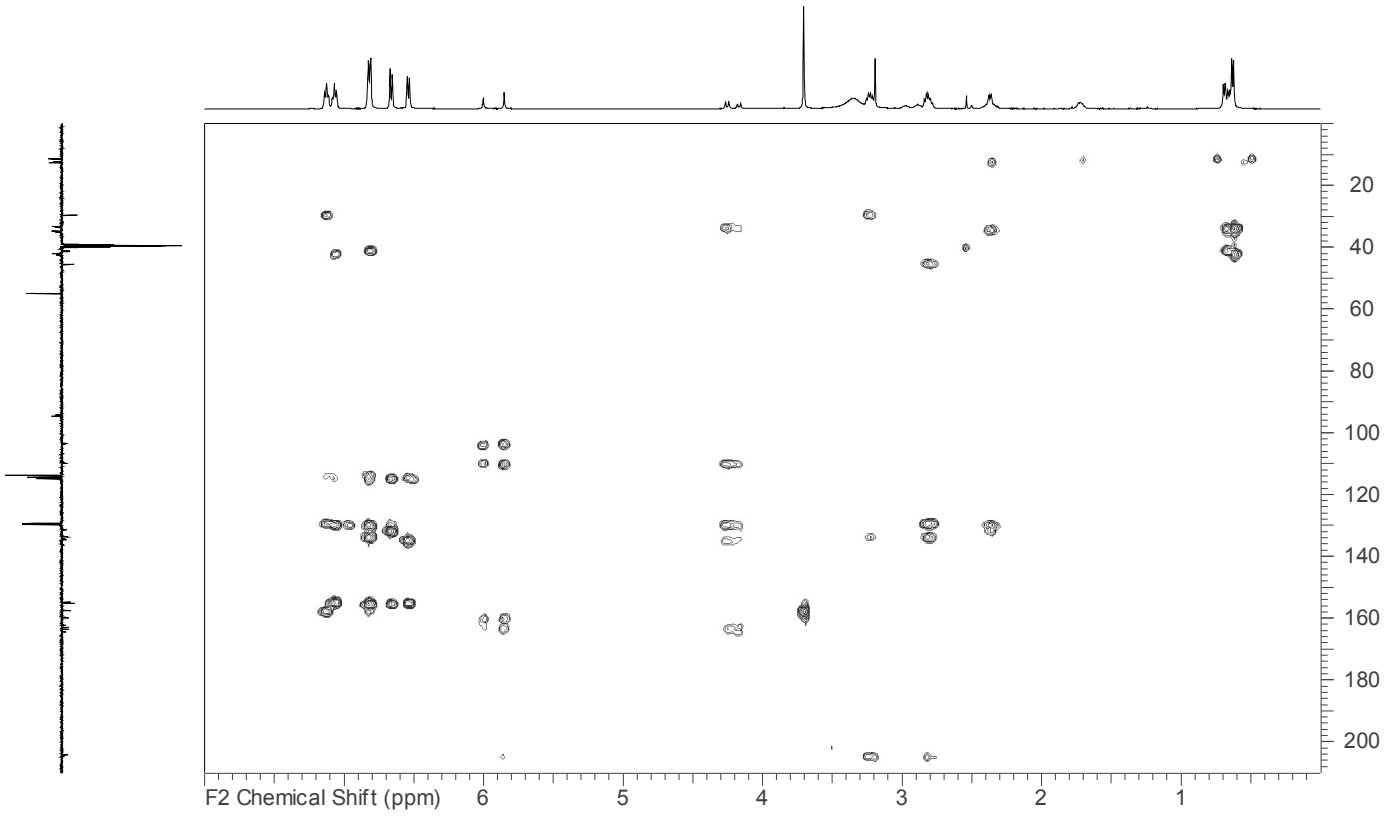

Figure S19. HMBC spectrum of compound **5** (500 MHz, DMSO-d_6)

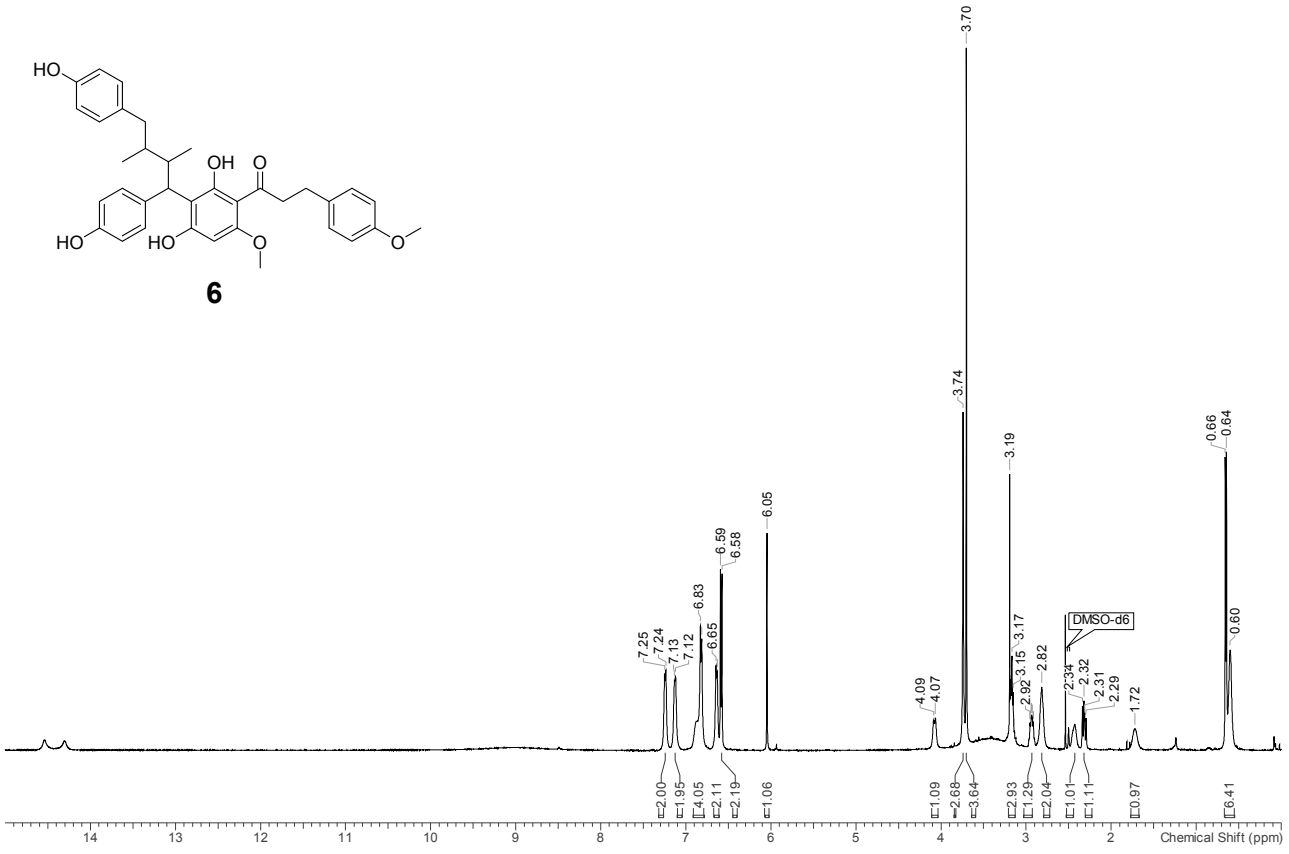

Figure S20. ^{1}H NMR spectrum of compound **6** (500 MHz, DMSO-d_6)

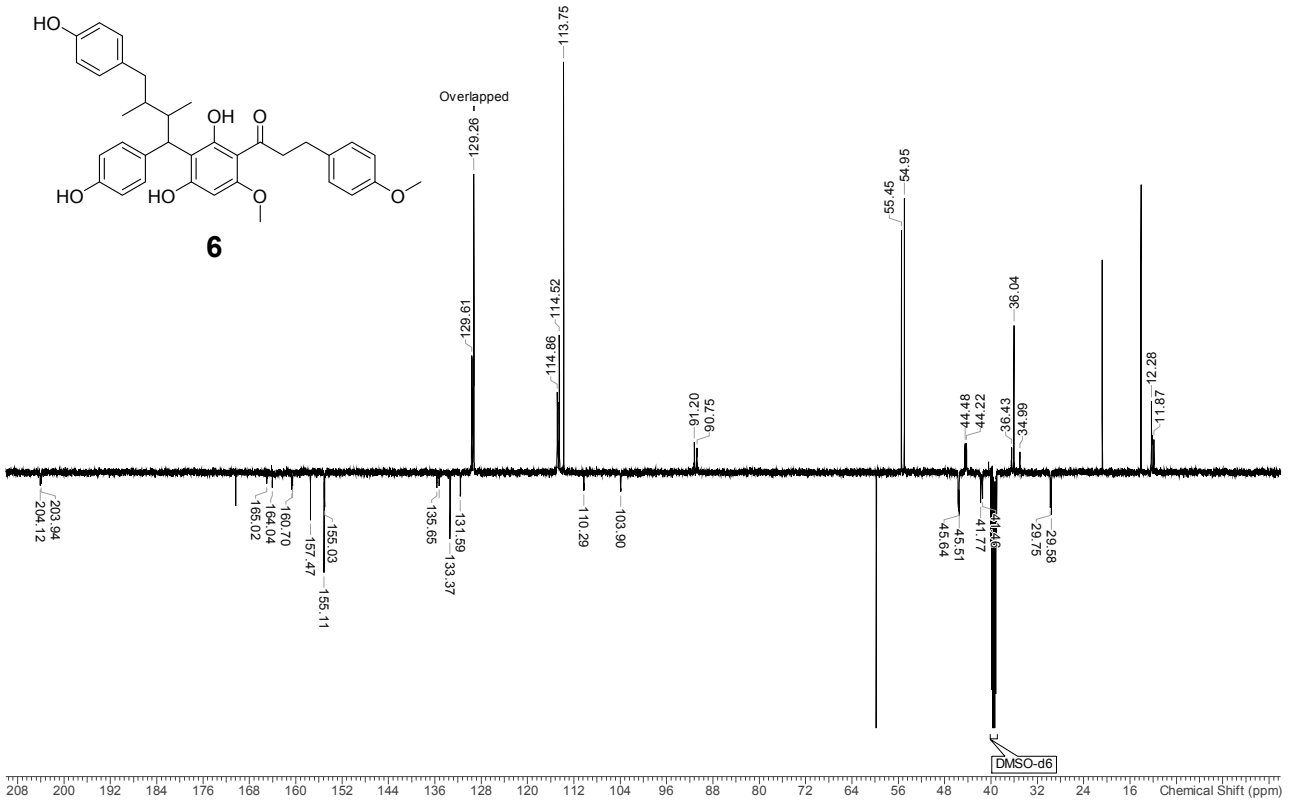

Figure S21. ^{13}C-DEPTq spectrum of compound **6** (125 MHz, DMSO-d_6)

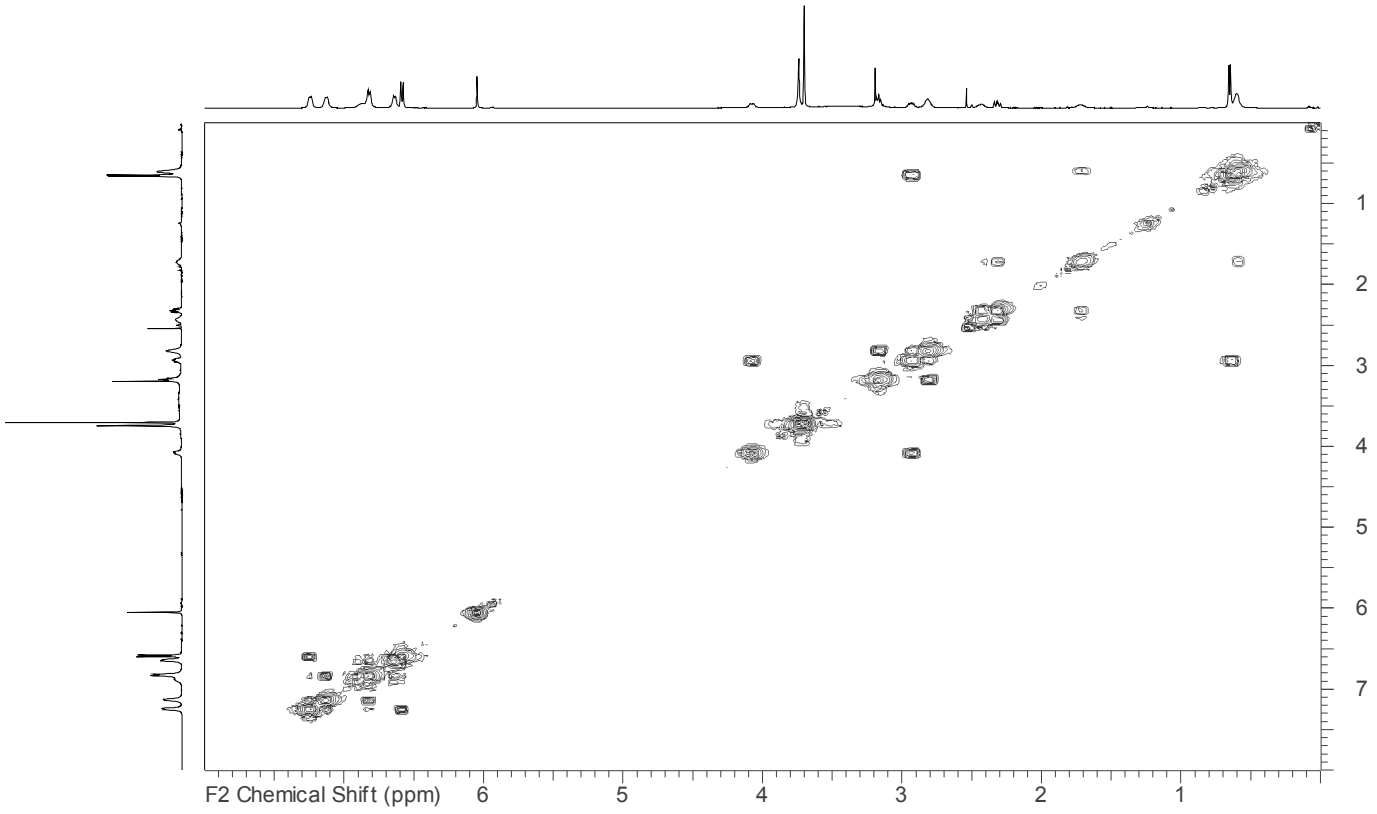

Figure S22. ^{1}H-^{1}H COSY spectrum of compound **6** (500 MHz, DMSO-d_6)

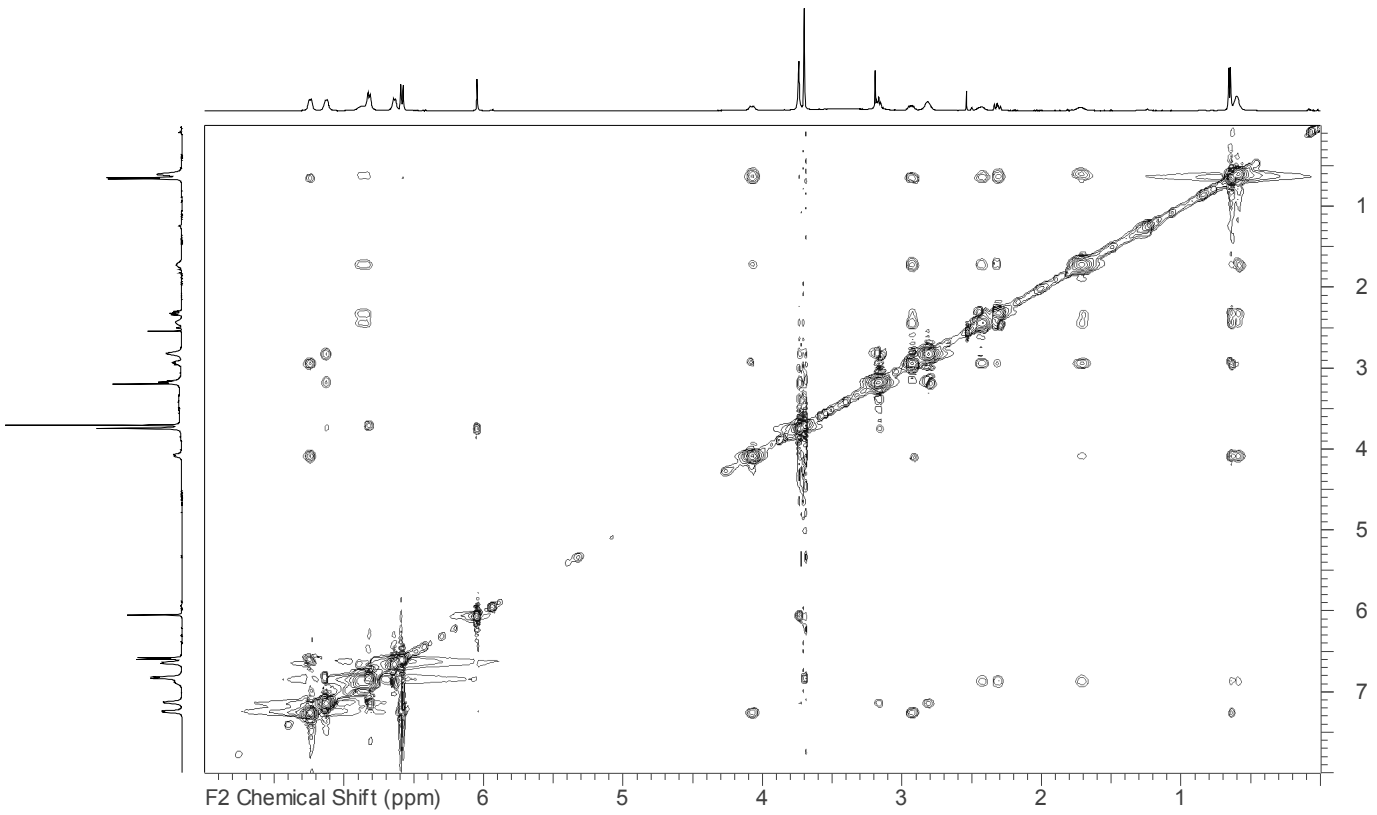

Figure S23. ^{1}H-^{1}H ROESY spectrum of compound **6** (500 MHz, DMSO-d_6)

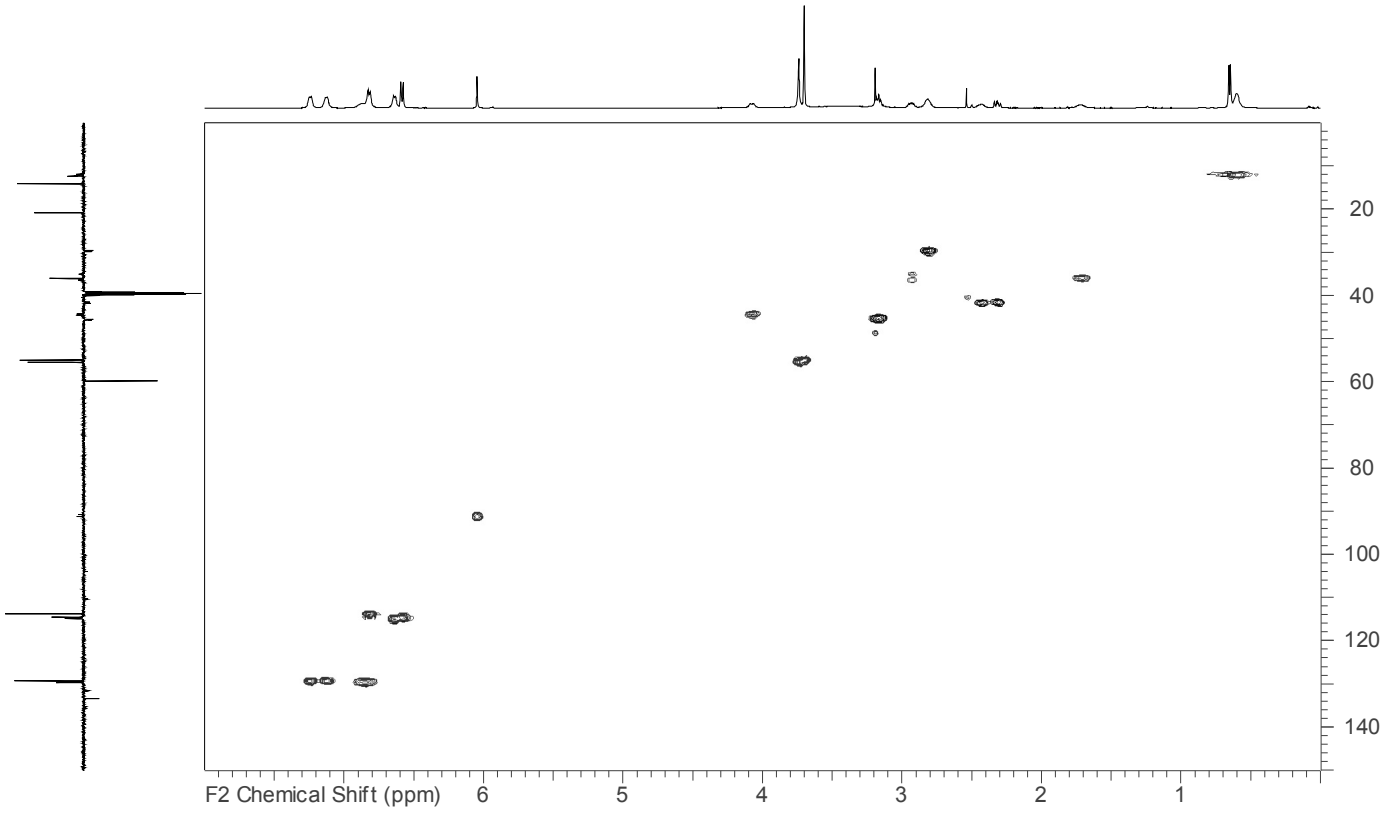

Figure S24. HSQC-DEPT spectrum of compound **6** (500 MHz, DMSO-d_6)

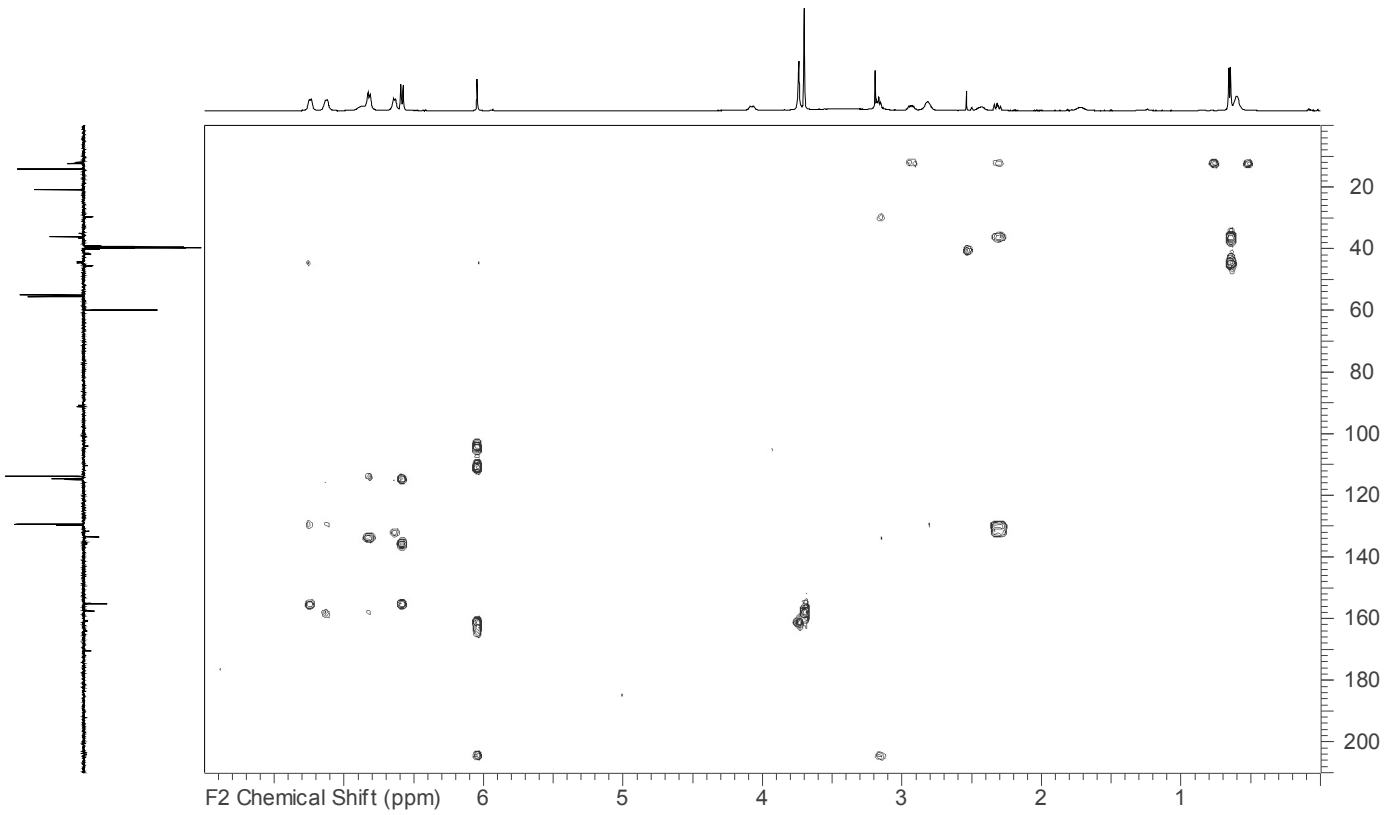

Figure S25. HMBC spectrum of compound **6** (500 MHz, DMSO-d_6)

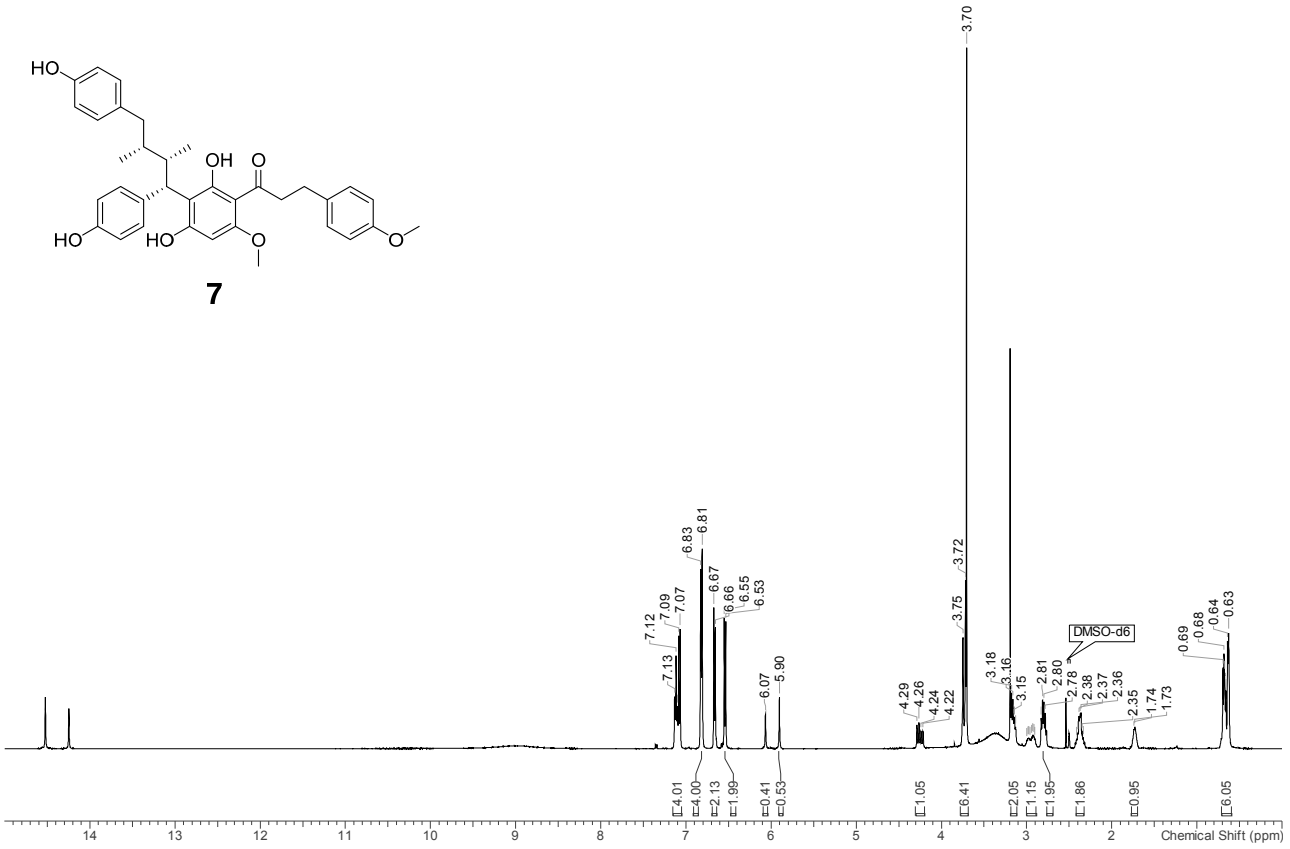

Figure S26. ^{1}H NMR spectrum of compound **7** (500 MHz, DMSO-d_6)

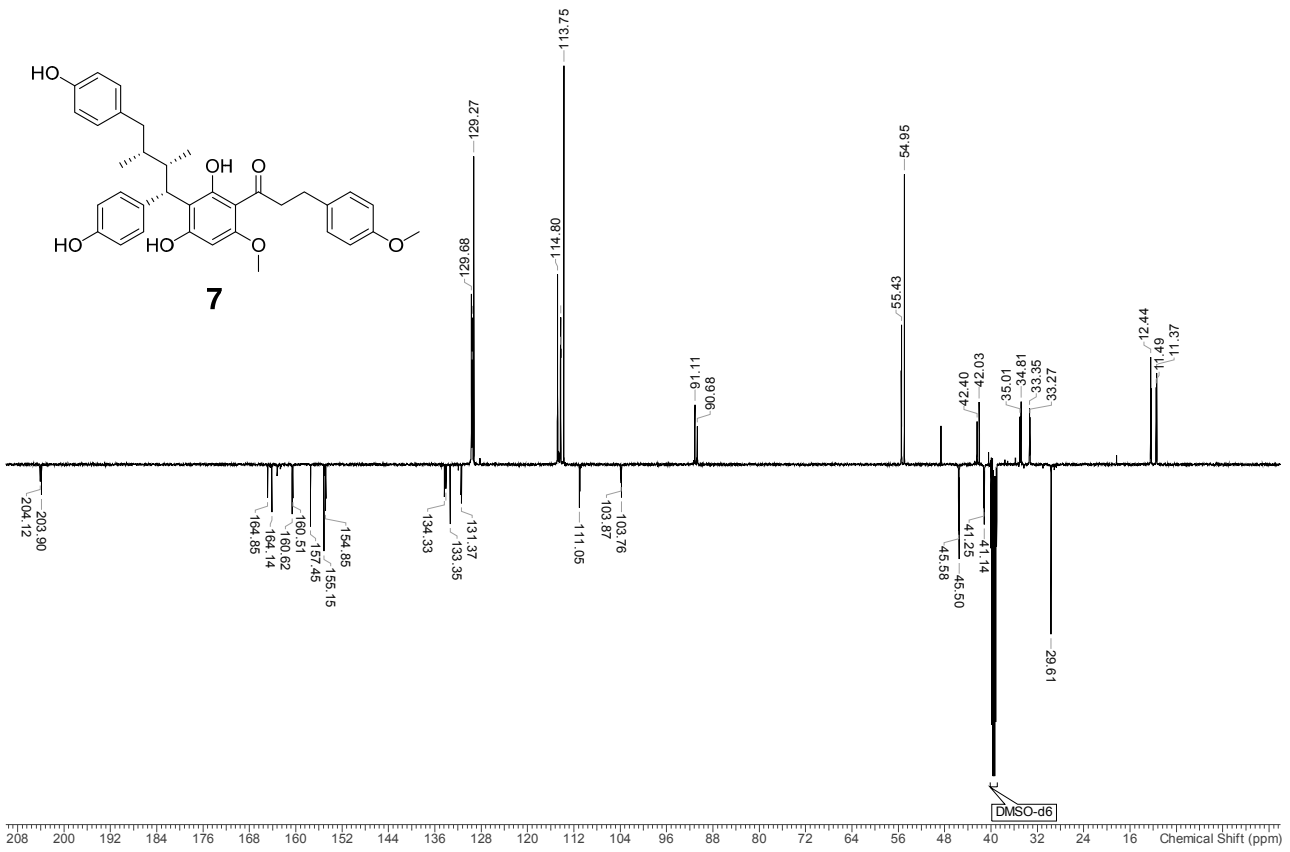

Figure S27. ^{13}C-DEPTq spectrum of compound **7** (125 MHz, DMSO-d_6)

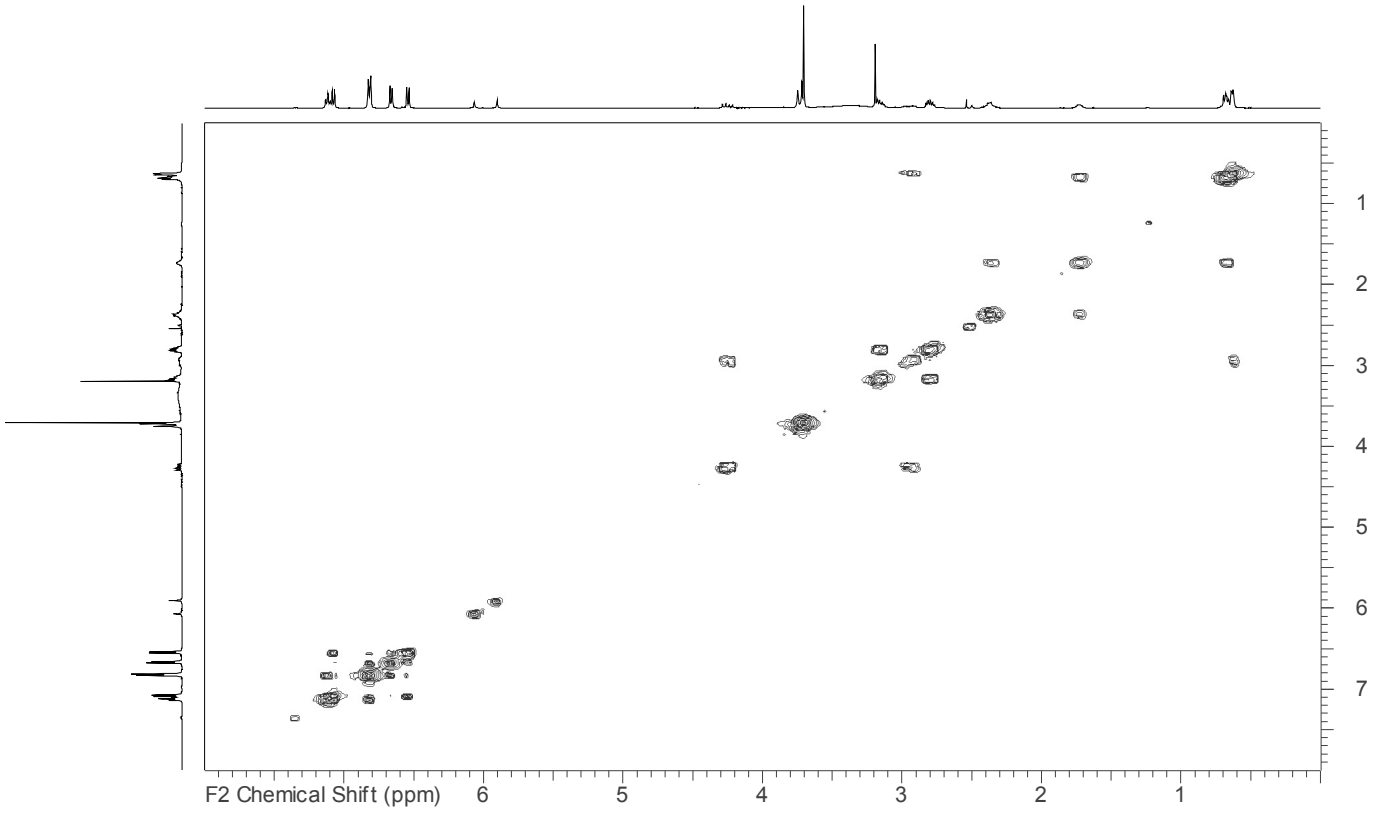

Figure S28. ^{1}H-^{1}H COSY spectrum of compound **7** (500 MHz, DMSO-d_6)

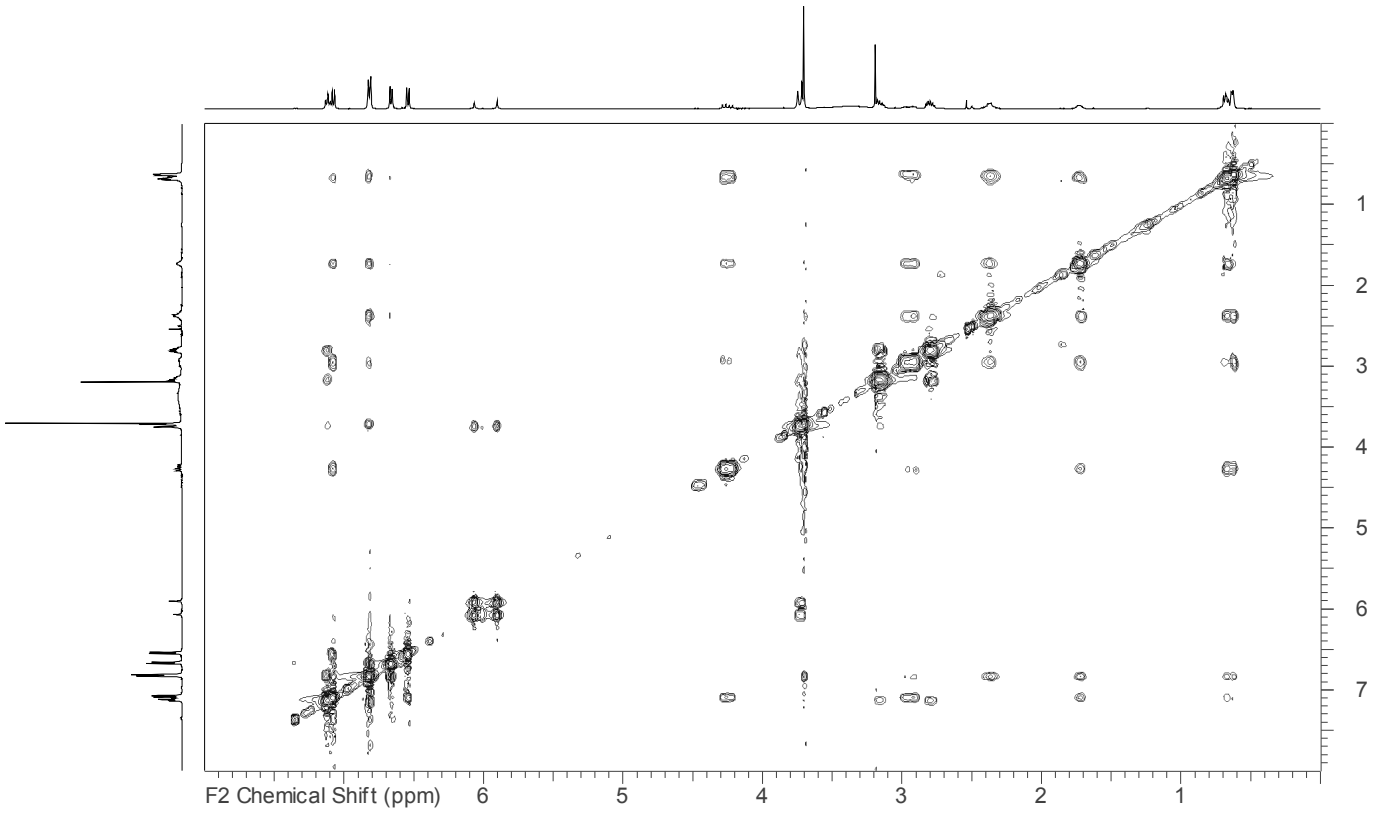

Figure S29. ^{1}H-^{1}H ROESY spectrum of compound **7** (500 MHz, DMSO-d_6)

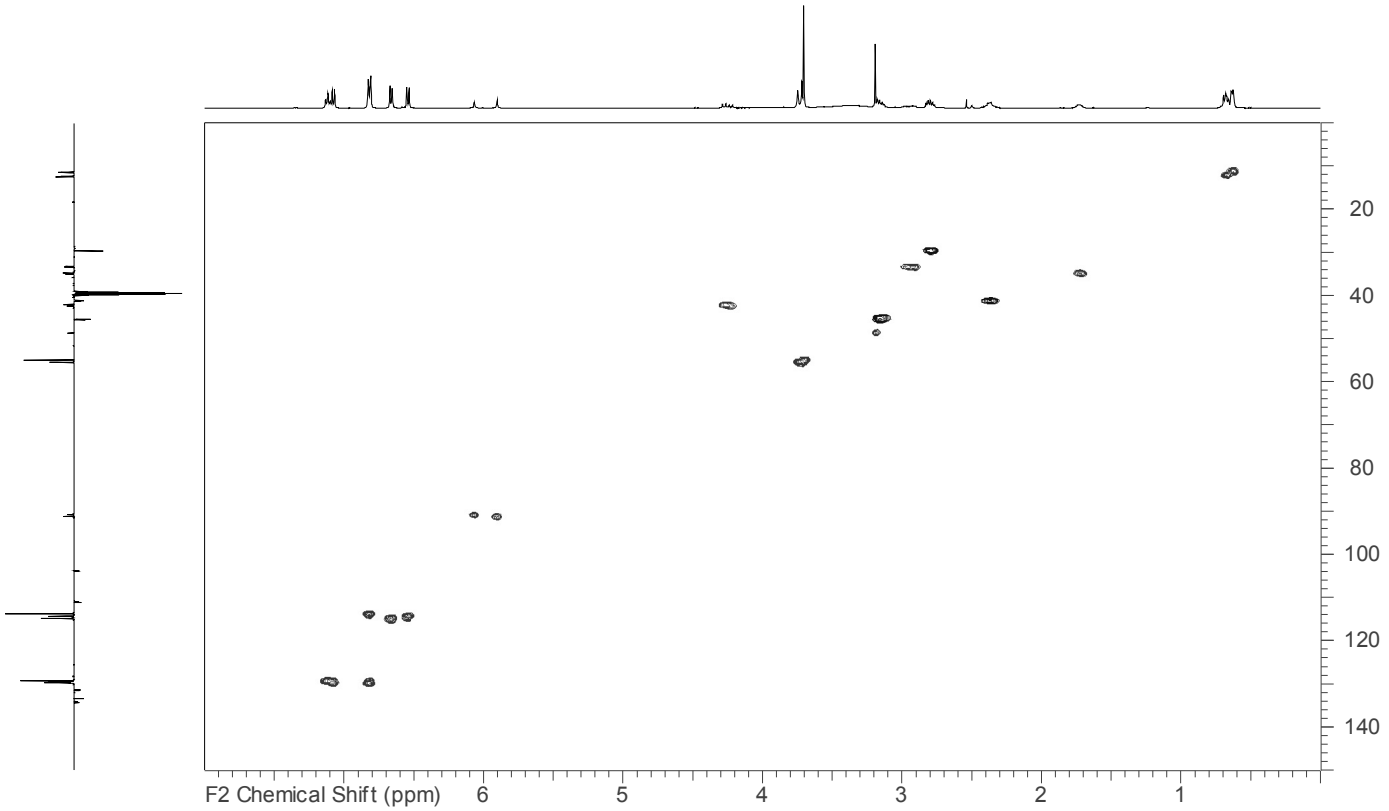

Figure S30. HSQC-DEPT spectrum of compound **7** (500 MHz, DMSO-d_6)

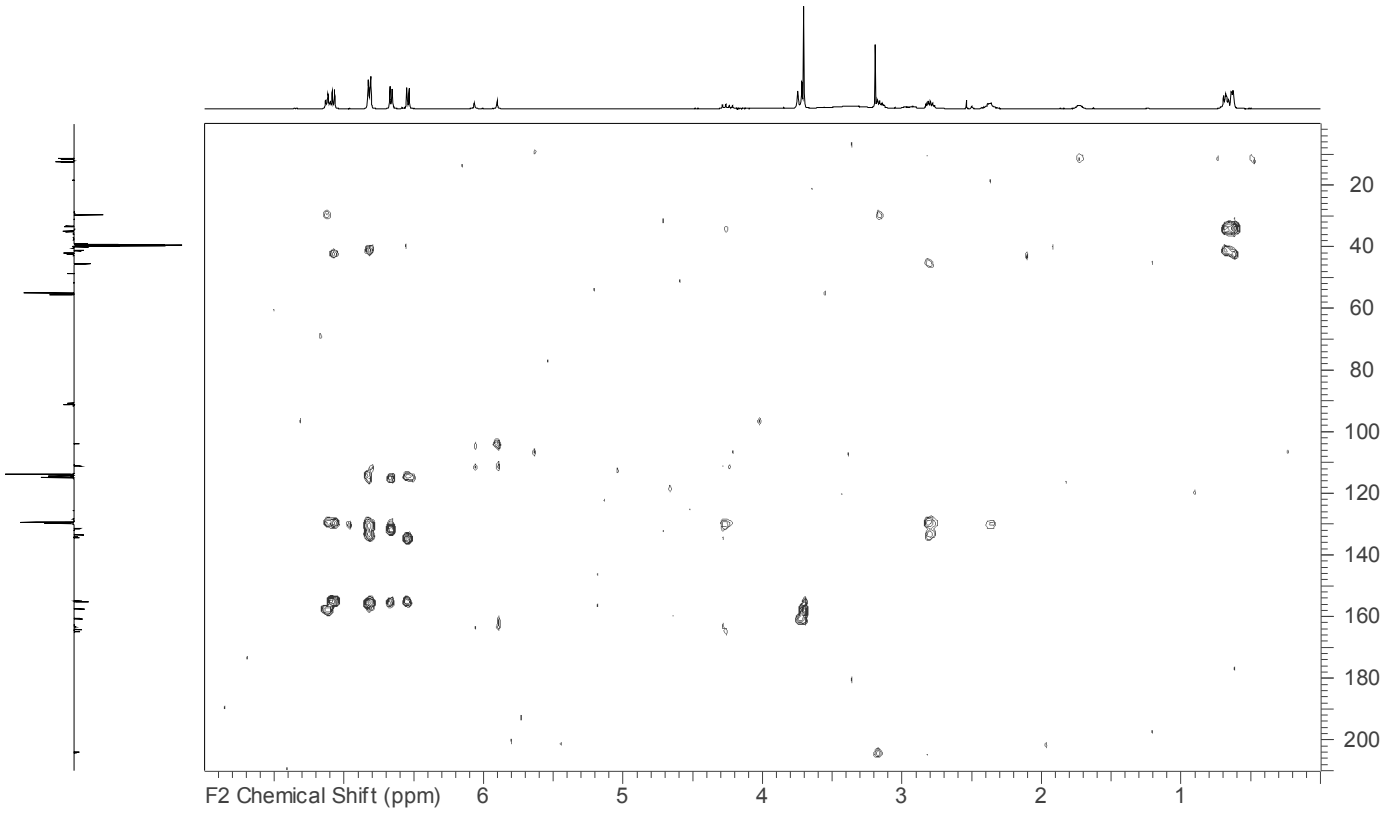

Figure S31. HMBC spectrum of compound **7** (500 MHz, DMSO-d_6)

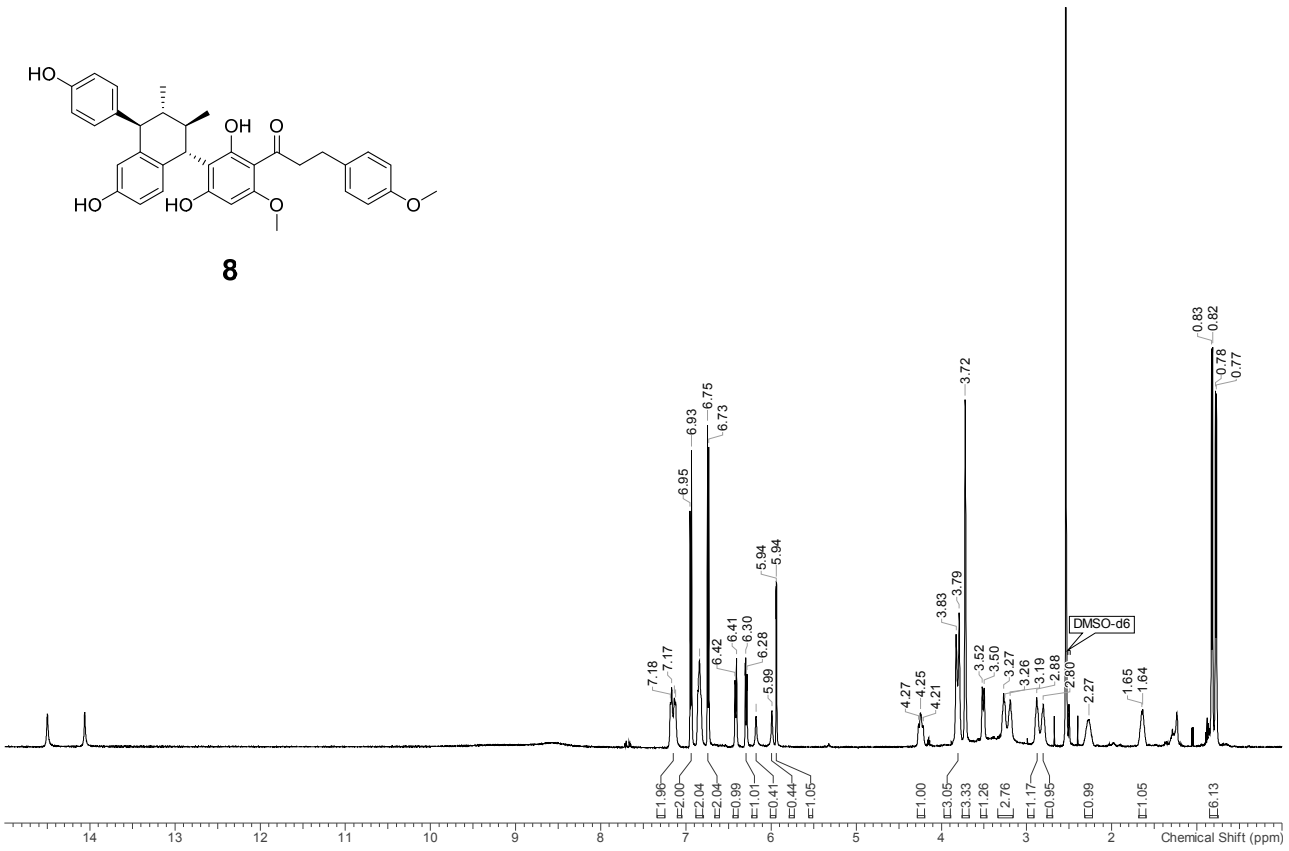

Figure S32. ^{1}H NMR spectrum of compound **8** (500 MHz, DMSO-d_6)

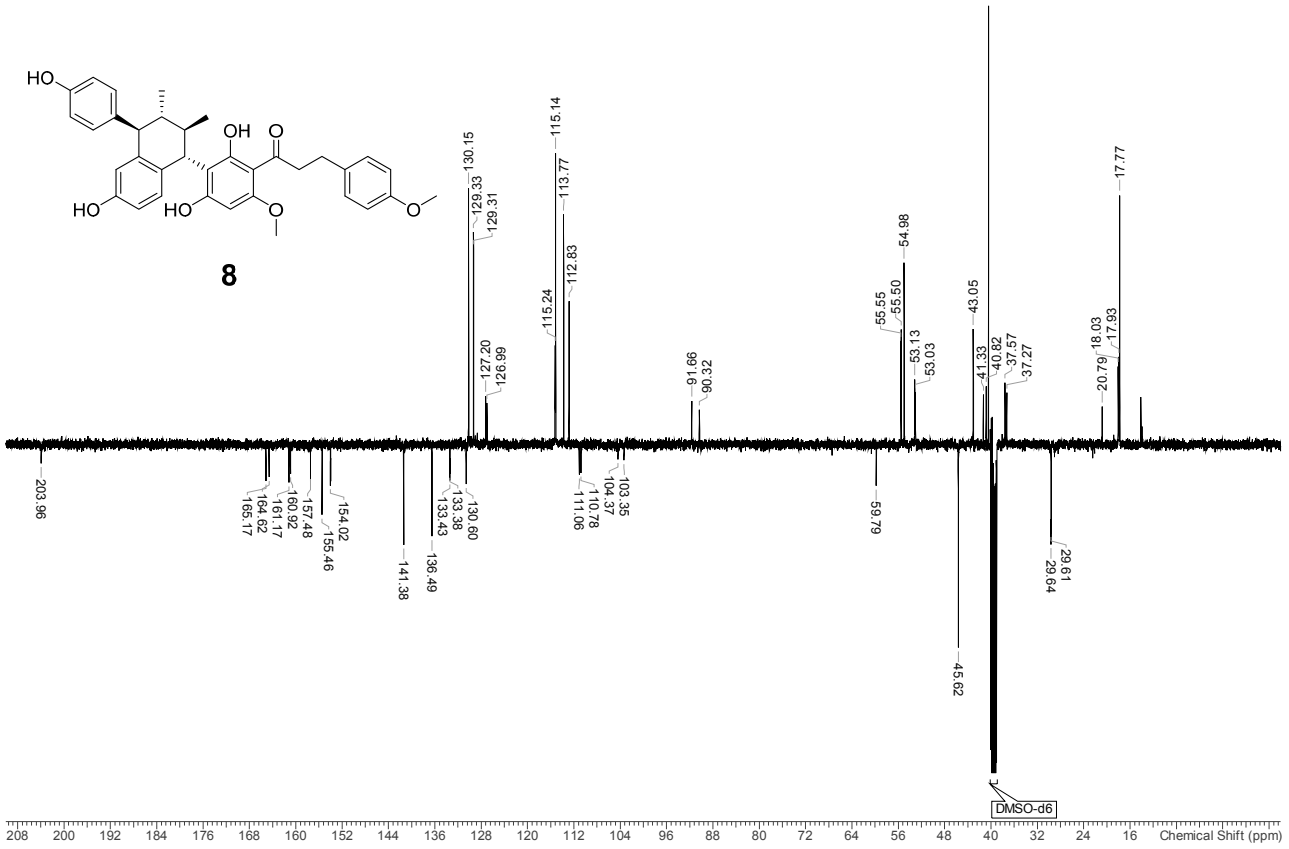

Figure S33. ^{13}C-DEPTq spectrum of compound **8** (125 MHz, DMSO-d_6)

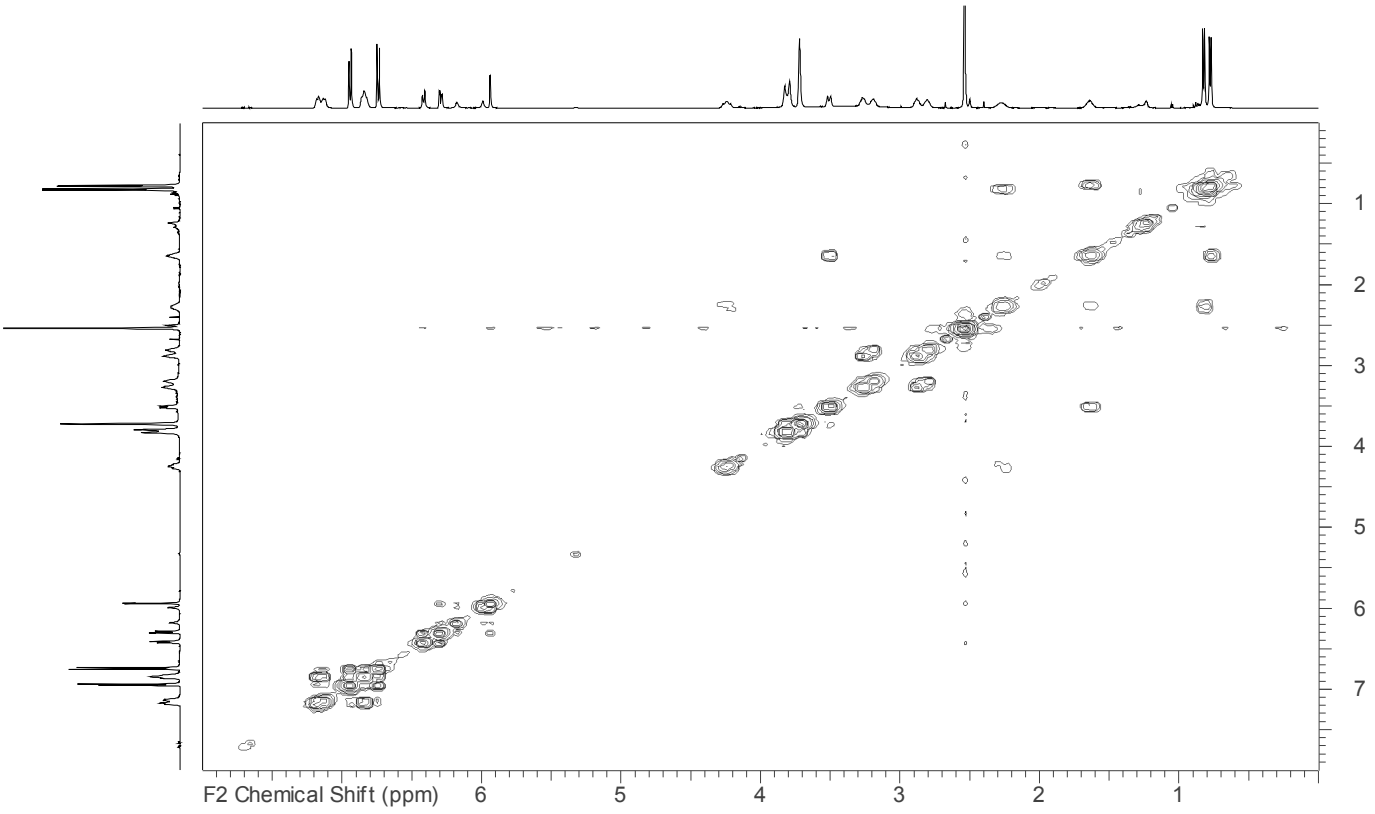

Figure S34. ^{1}H-^{1}H COSY spectrum of compound **8** (500 MHz, DMSO-d_6)

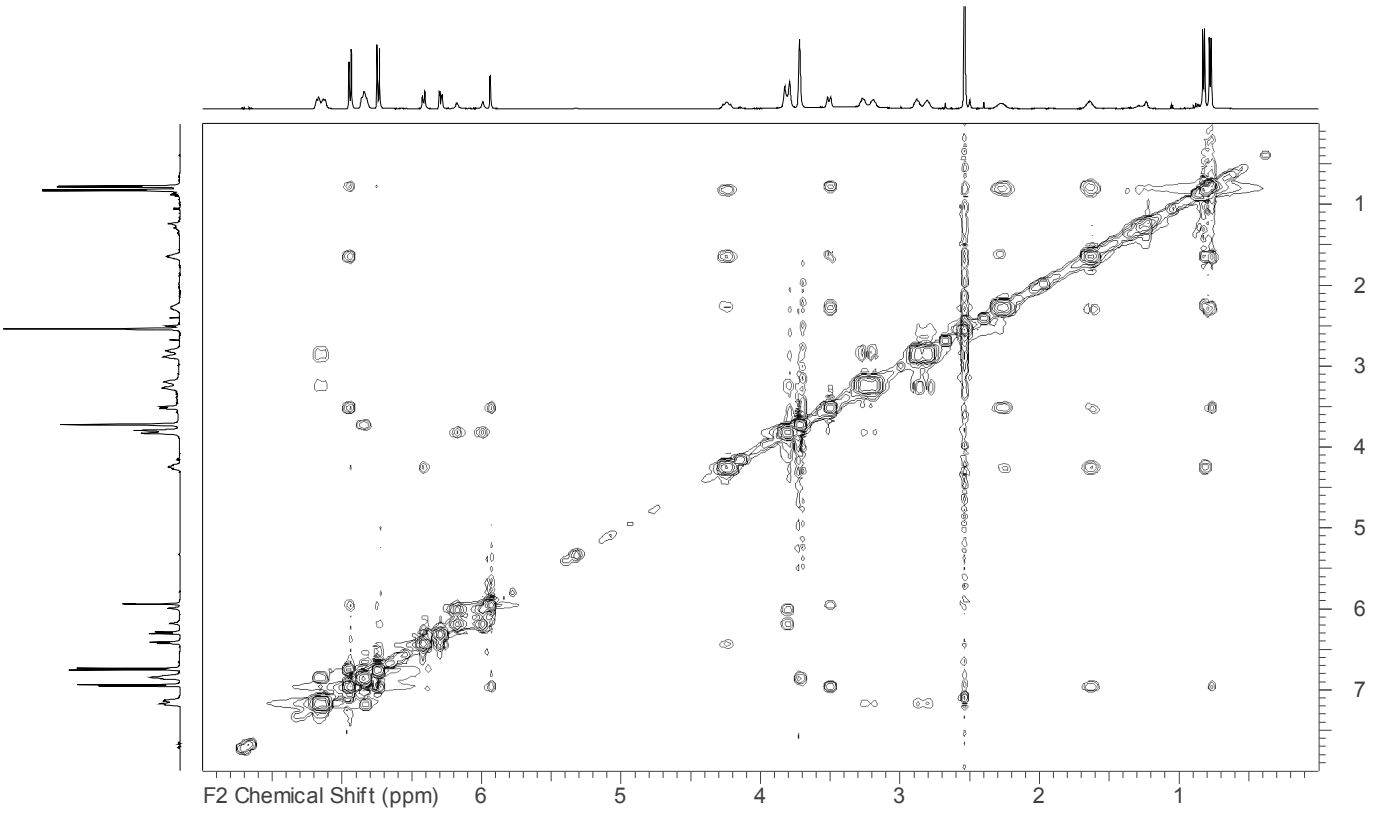

Figure S35. ^{1}H-^{1}H ROESY spectrum of compound **8** (500 MHz, DMSO-d_6)

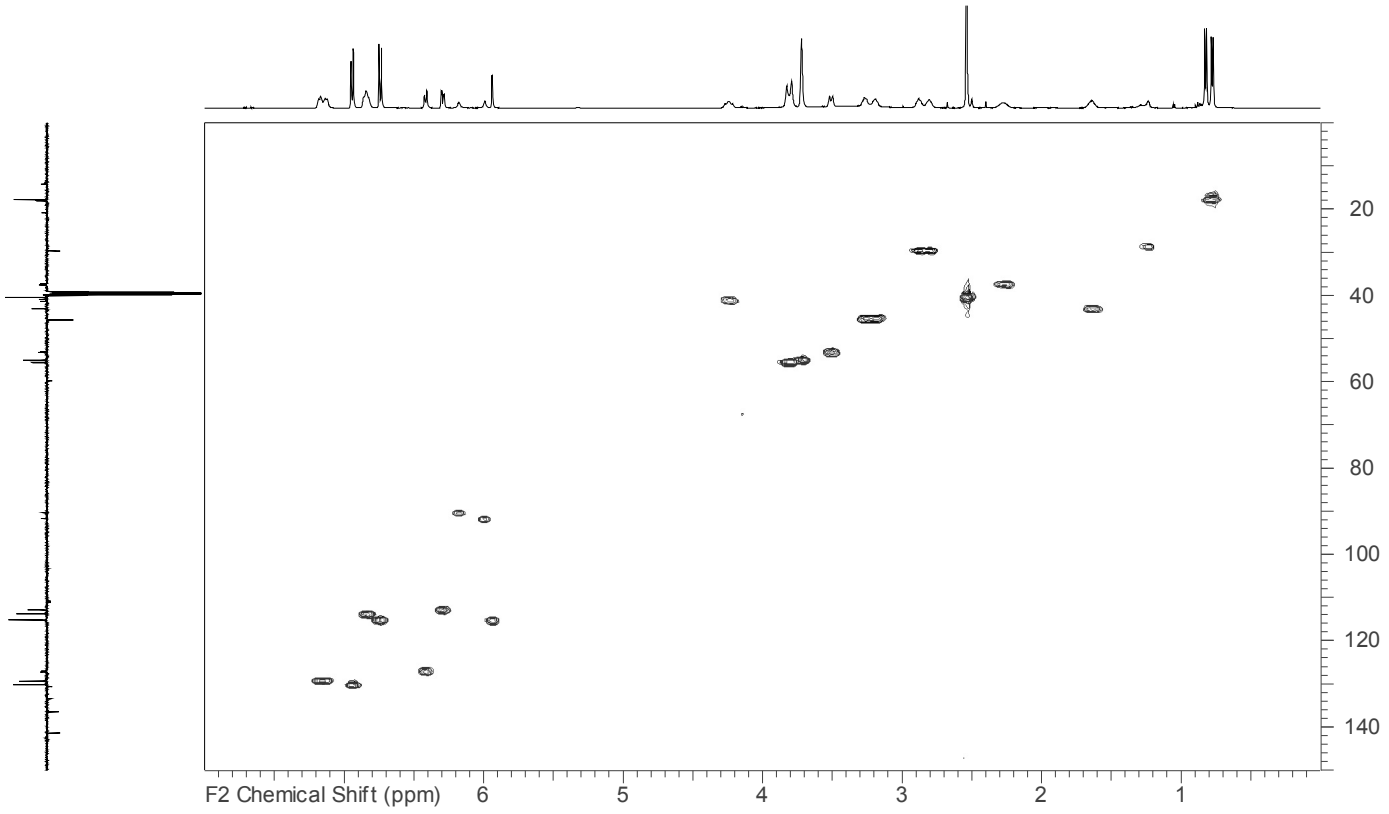

Figure S36. HSQC-DEPT spectrum of compound **8** (500 MHz, DMSO-d_6)

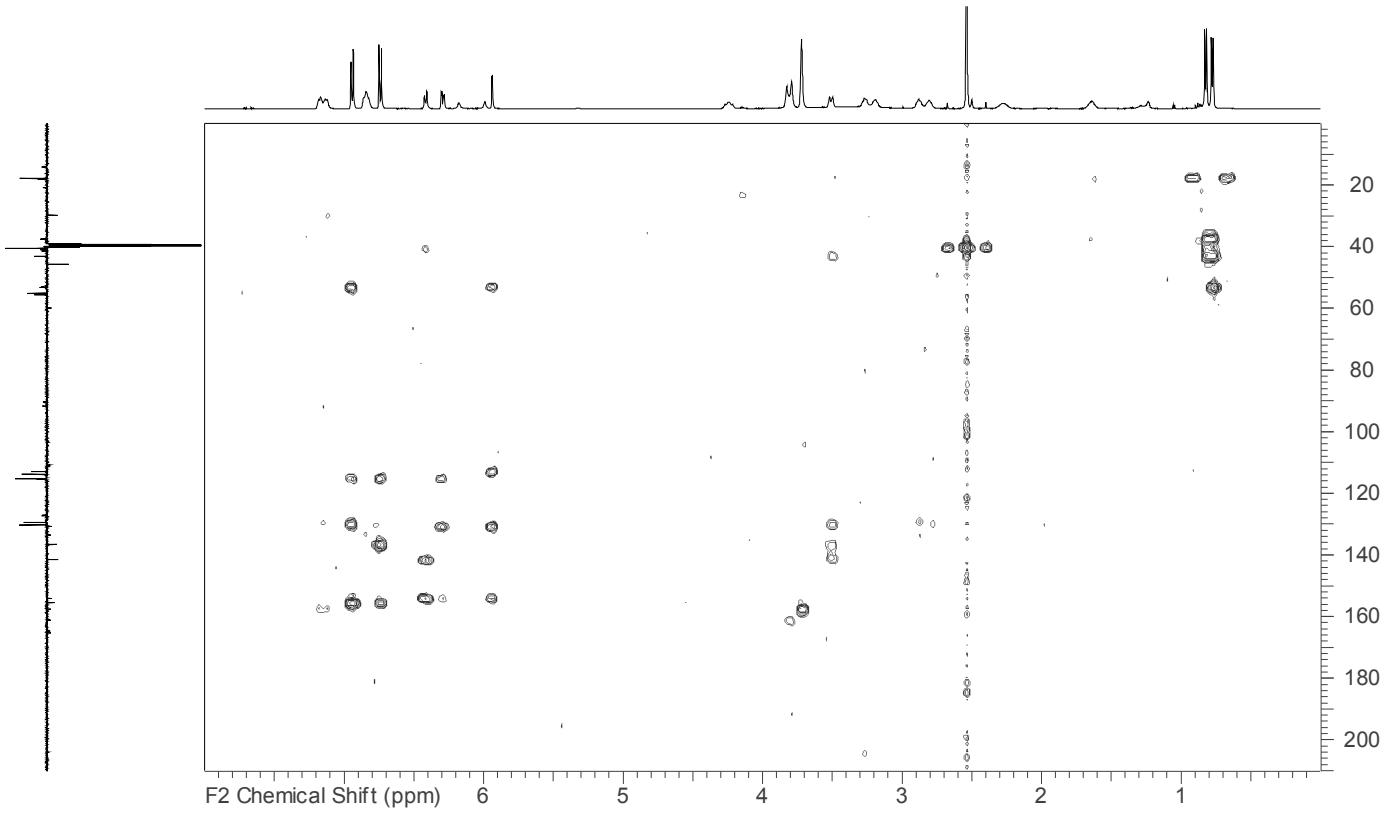

Figure S37. HMBC spectrum of compound **8** (500 MHz, DMSO-d_6)

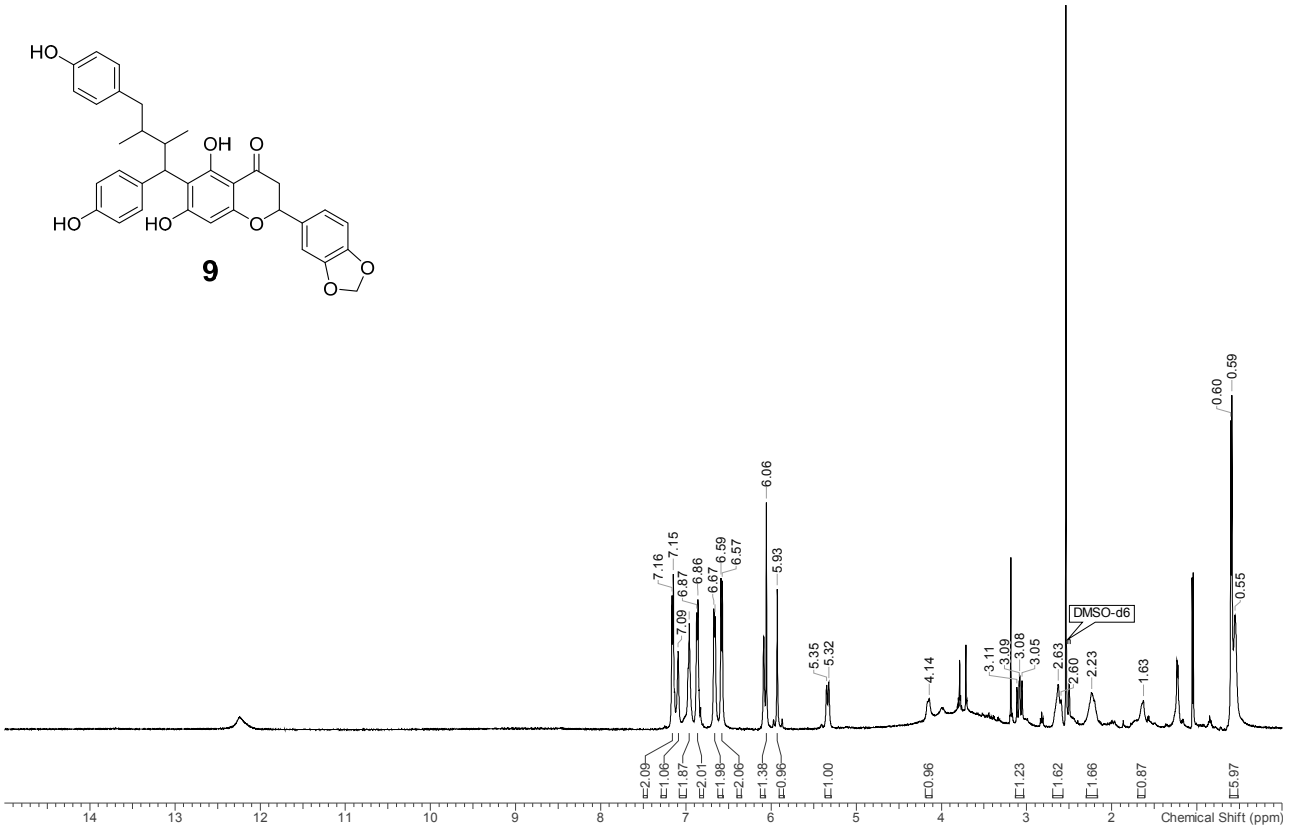

Figure S38. ^{1}H NMR spectrum of compound **9** (500 MHz, DMSO-d_6)

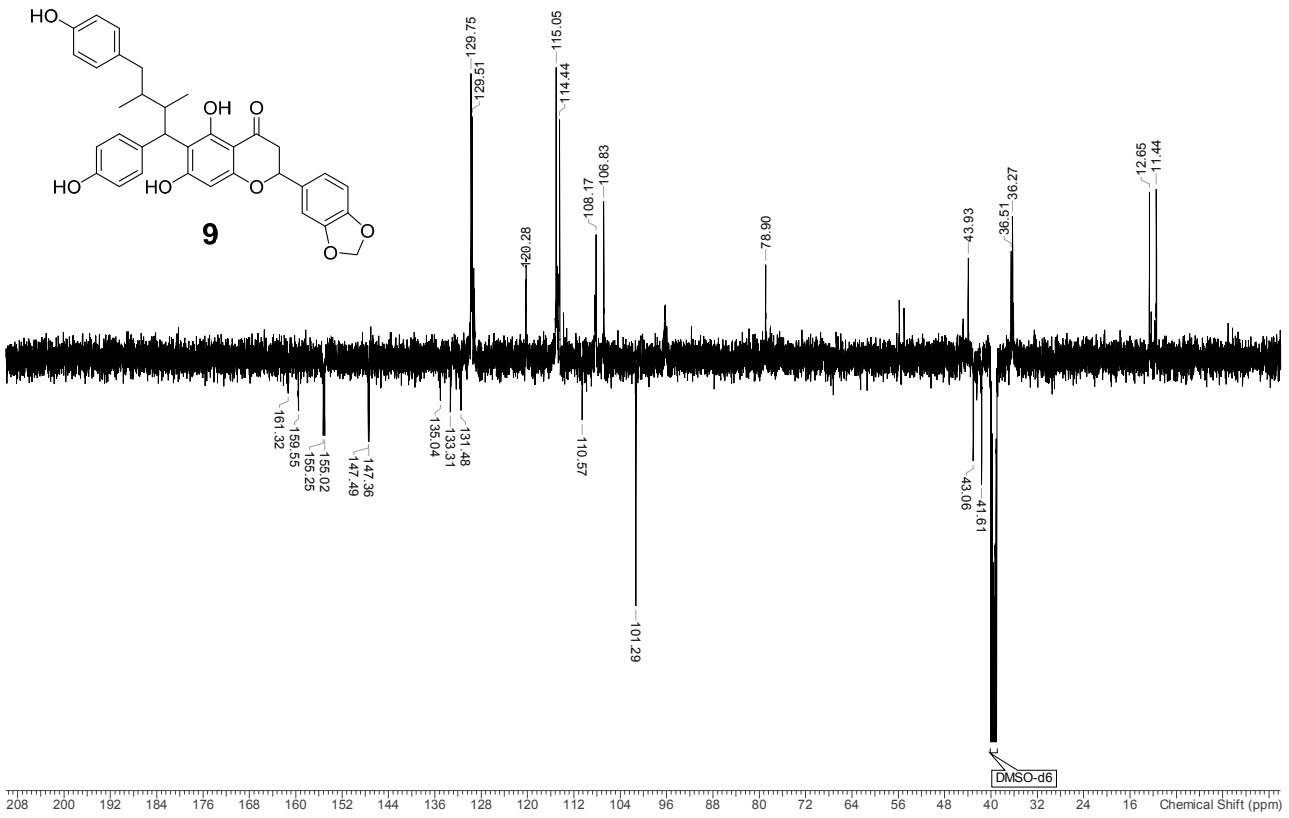

Figure S39. ^{13}C-DEPTq spectrum of compound **9** (125 MHz, DMSO-d_6)

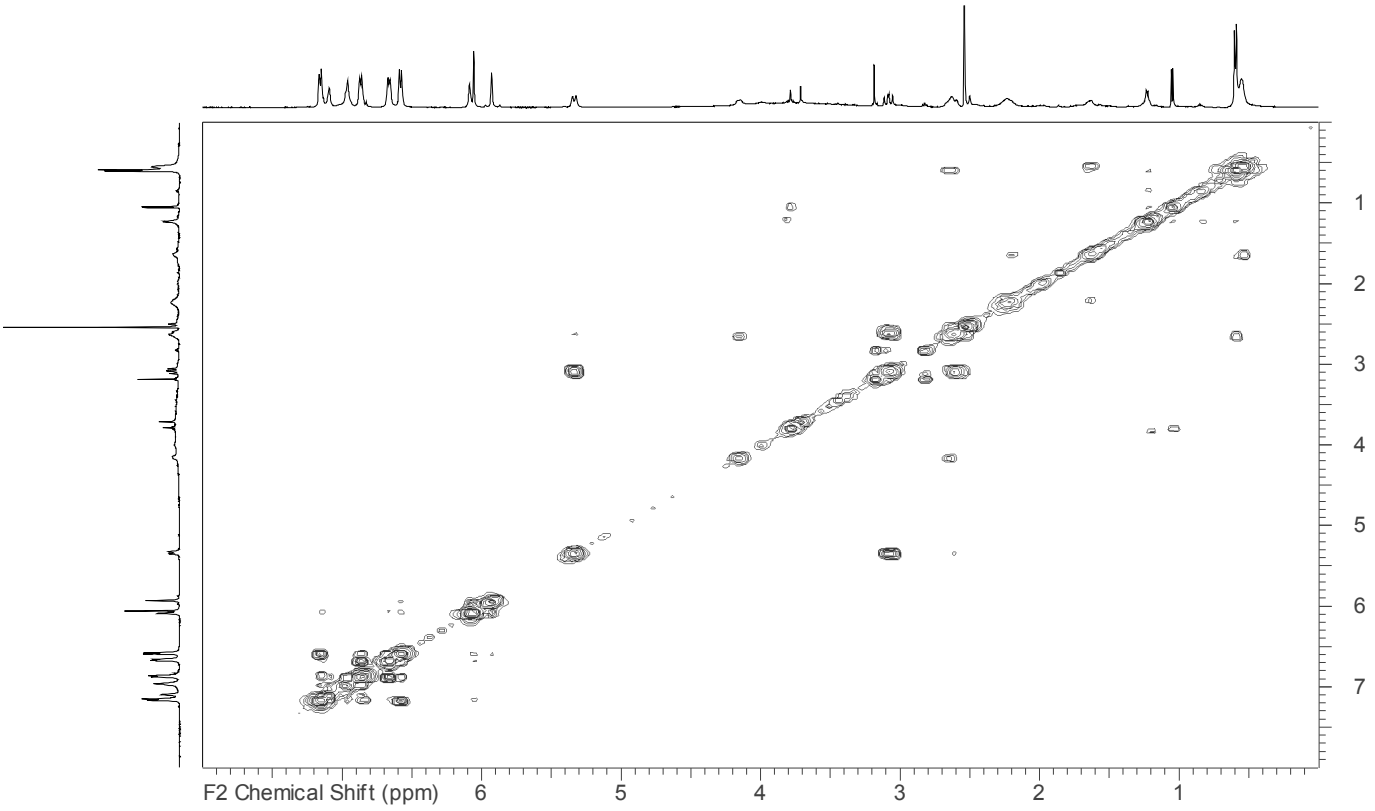

Figure S40. ^{1}H-^{1}H COSY spectrum of compound **9** (500 MHz, DMSO-d_6)

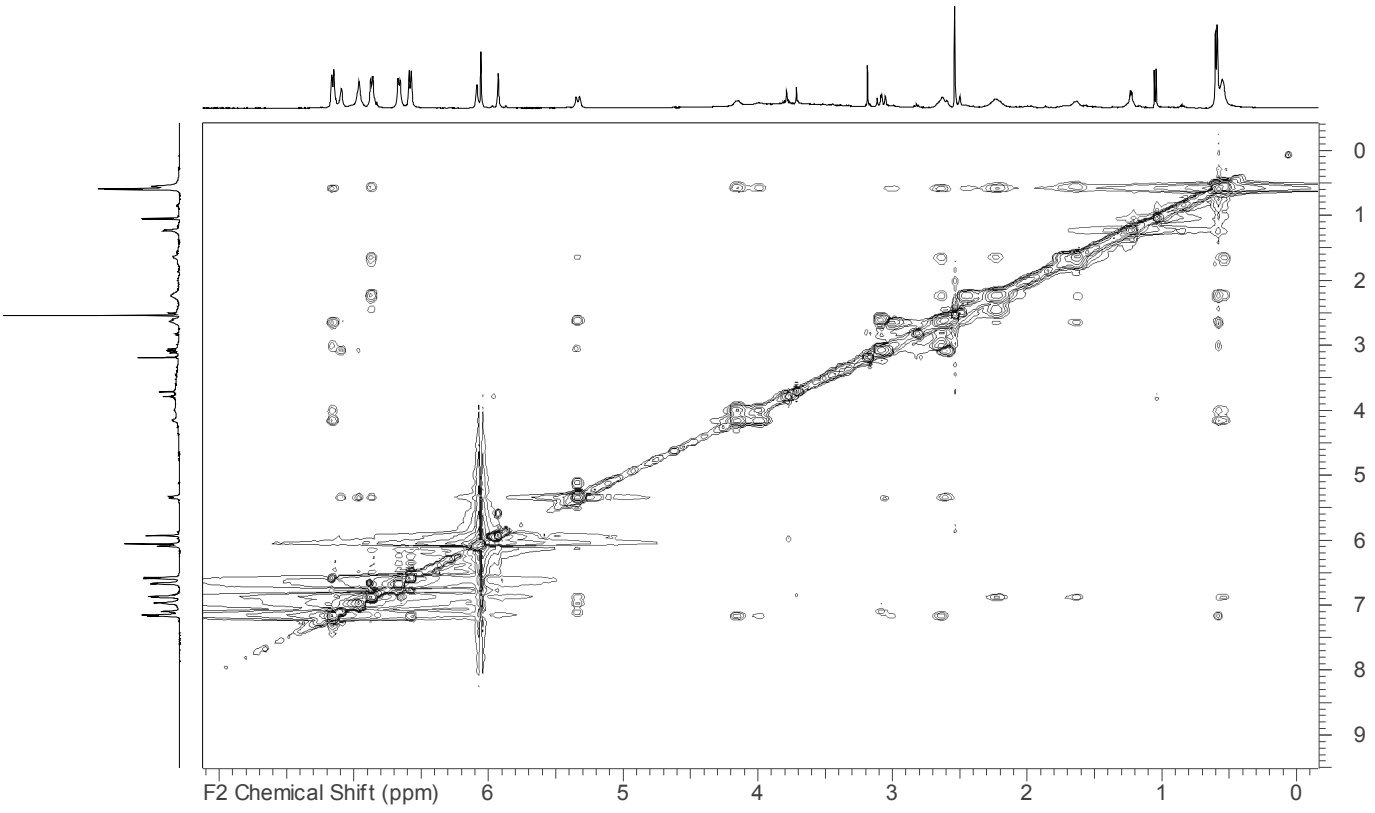

Figure S41. ^{1}H-^{1}H ROESY spectrum of compound **9** (500 MHz, DMSO-d_6)

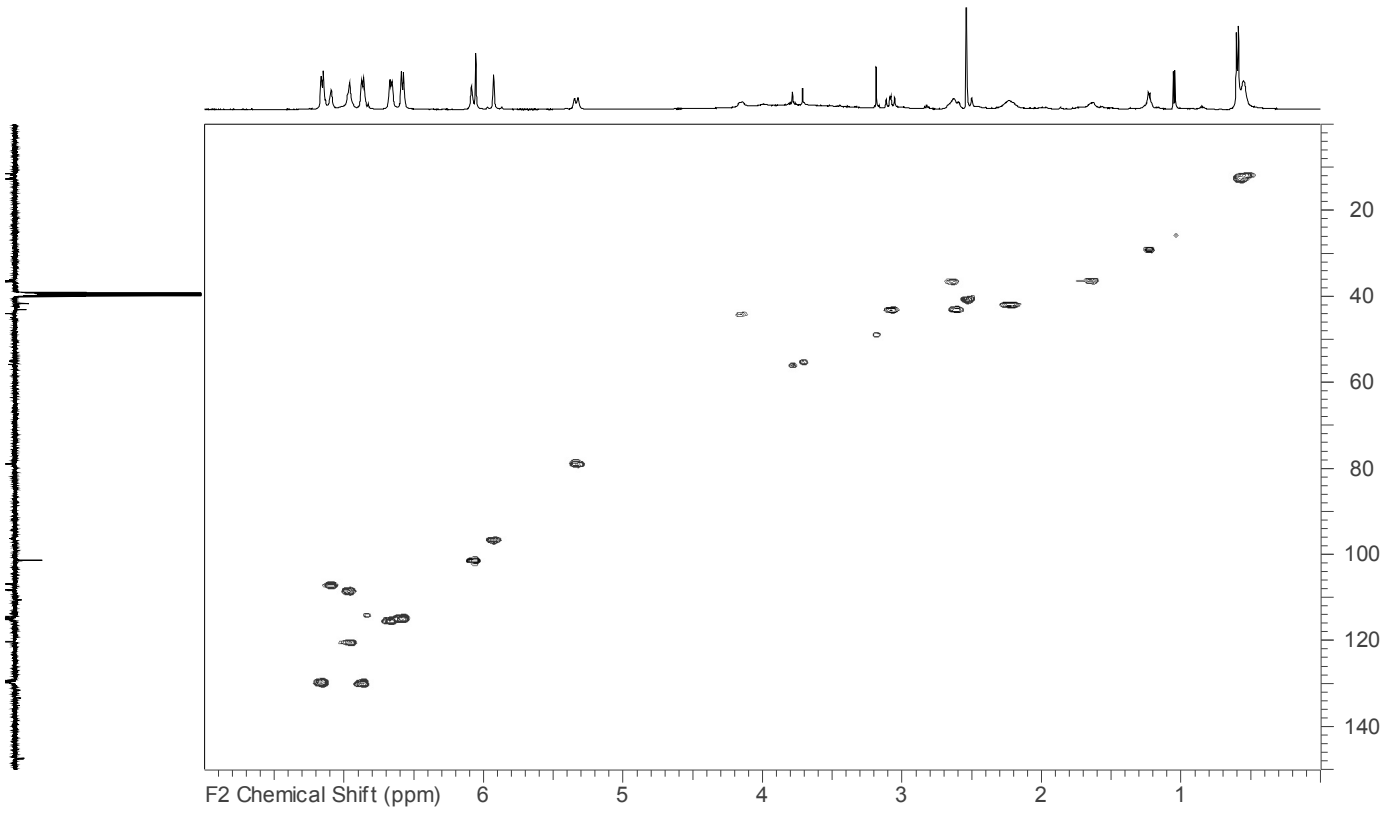

Figure S42. HSQC-DEPT spectrum of compound **9** (500 MHz, DMSO-d_6)

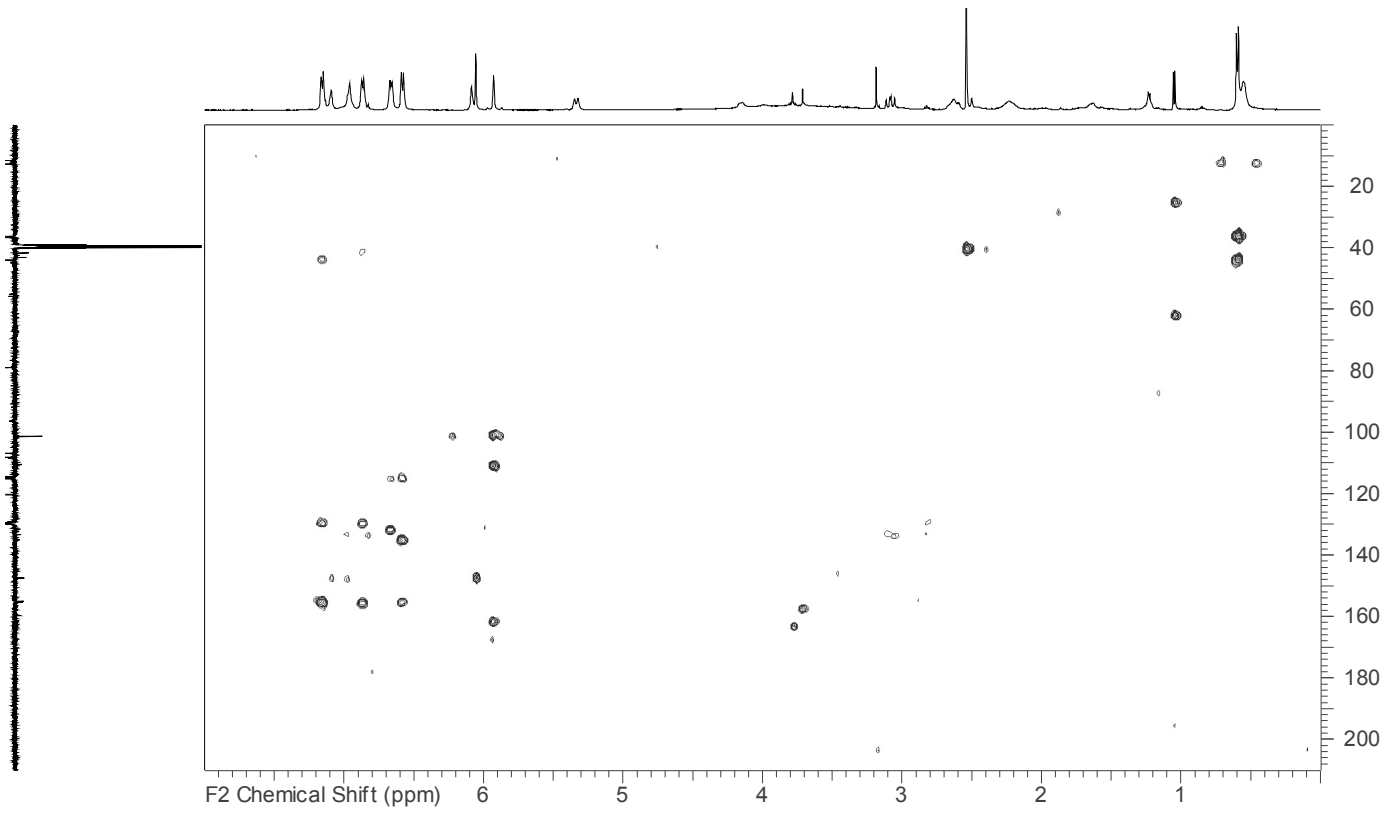

Figure S43. HMBC spectrum of compound **9** (500 MHz, DMSO-d_6)

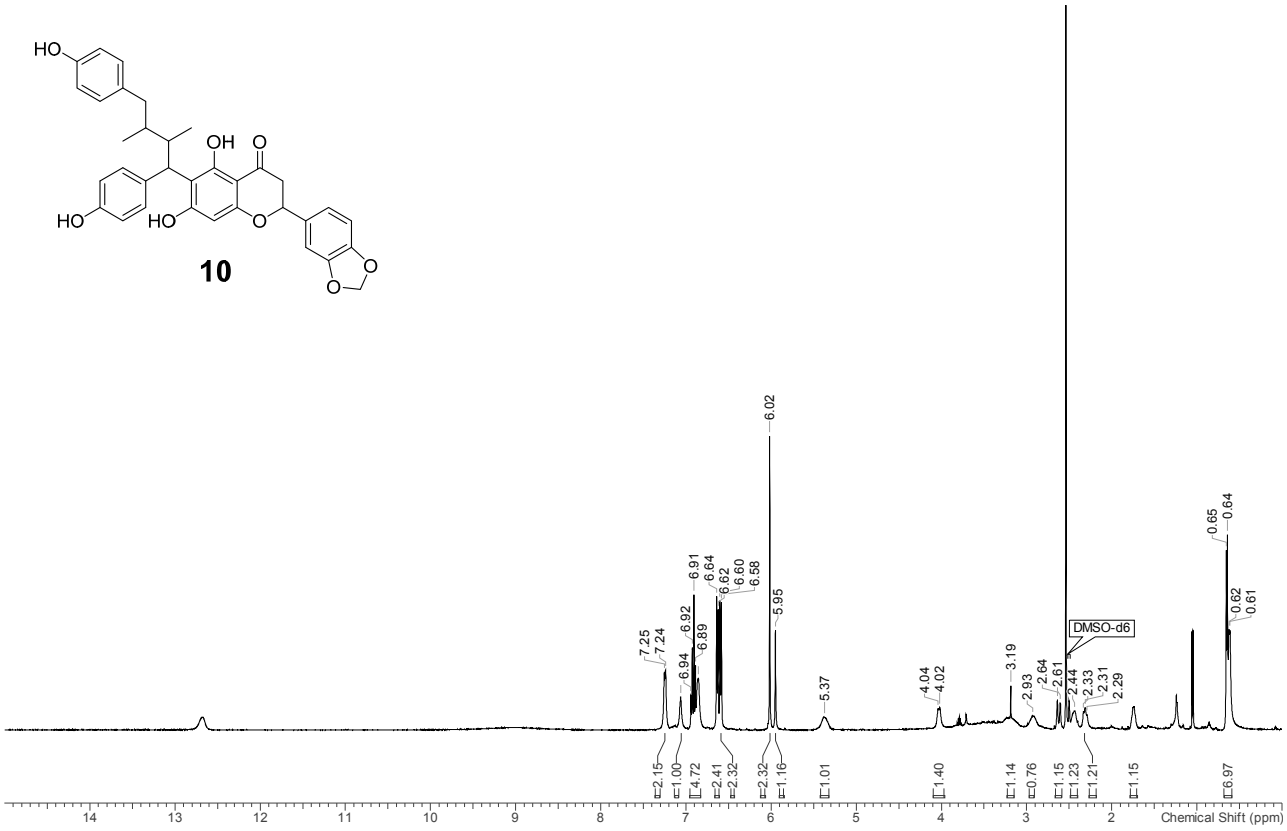

Figure S44. ^{1}H NMR spectrum of compound **10** (500 MHz, DMSO-d_6)

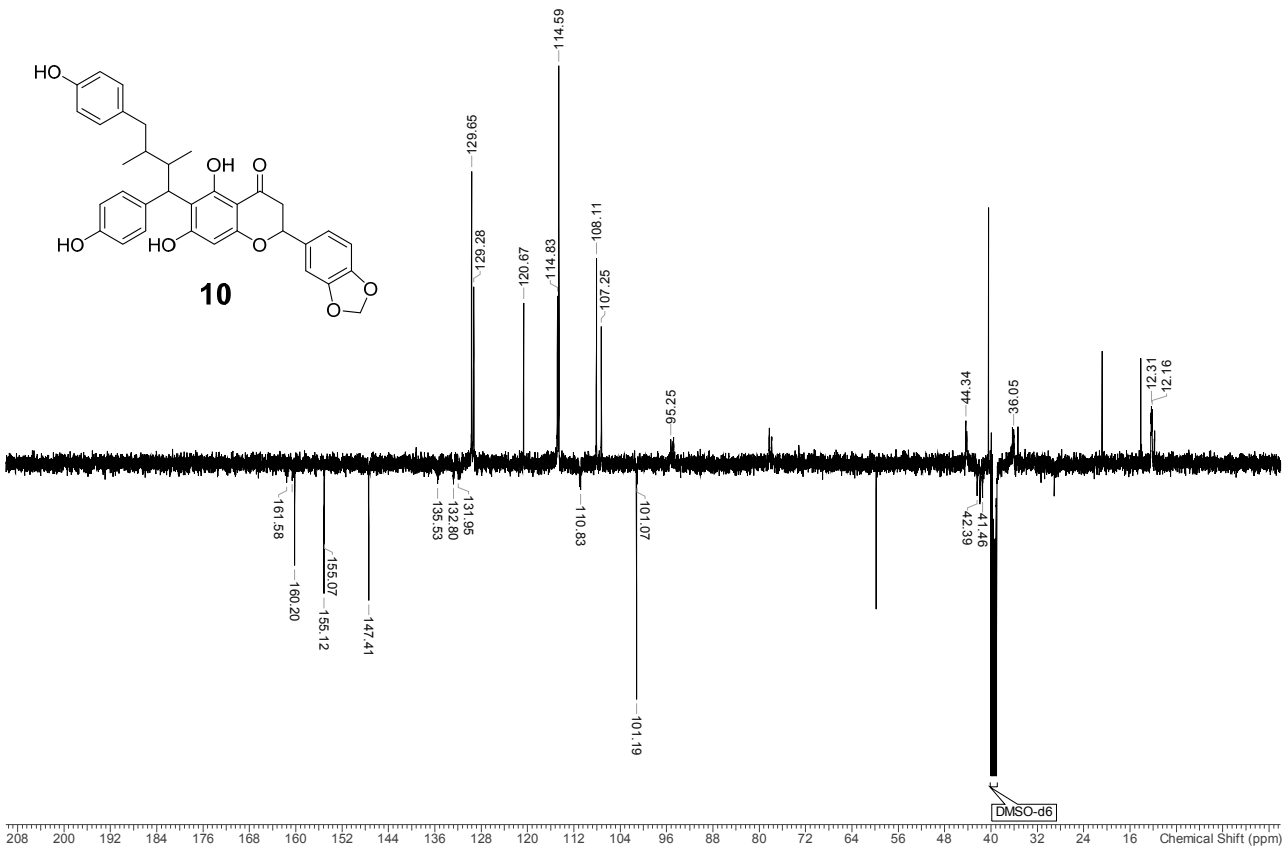

Figure S45. ^{13}C-DEPTq spectrum of compound **10** (125 MHz, DMSO-d_6)

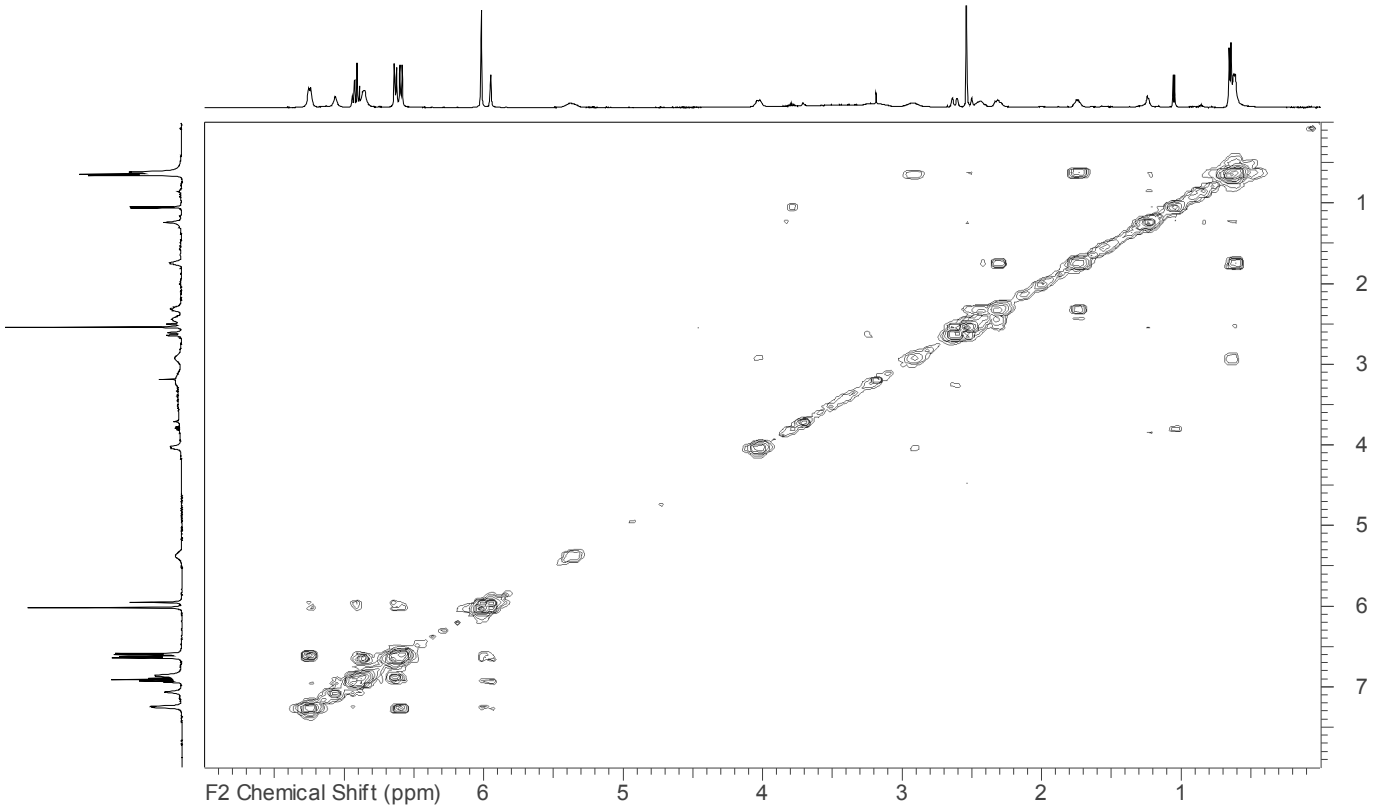

Figure S46. ^{1}H-^{1}H COSY spectrum of compound **10** (500 MHz, DMSO-d_6)

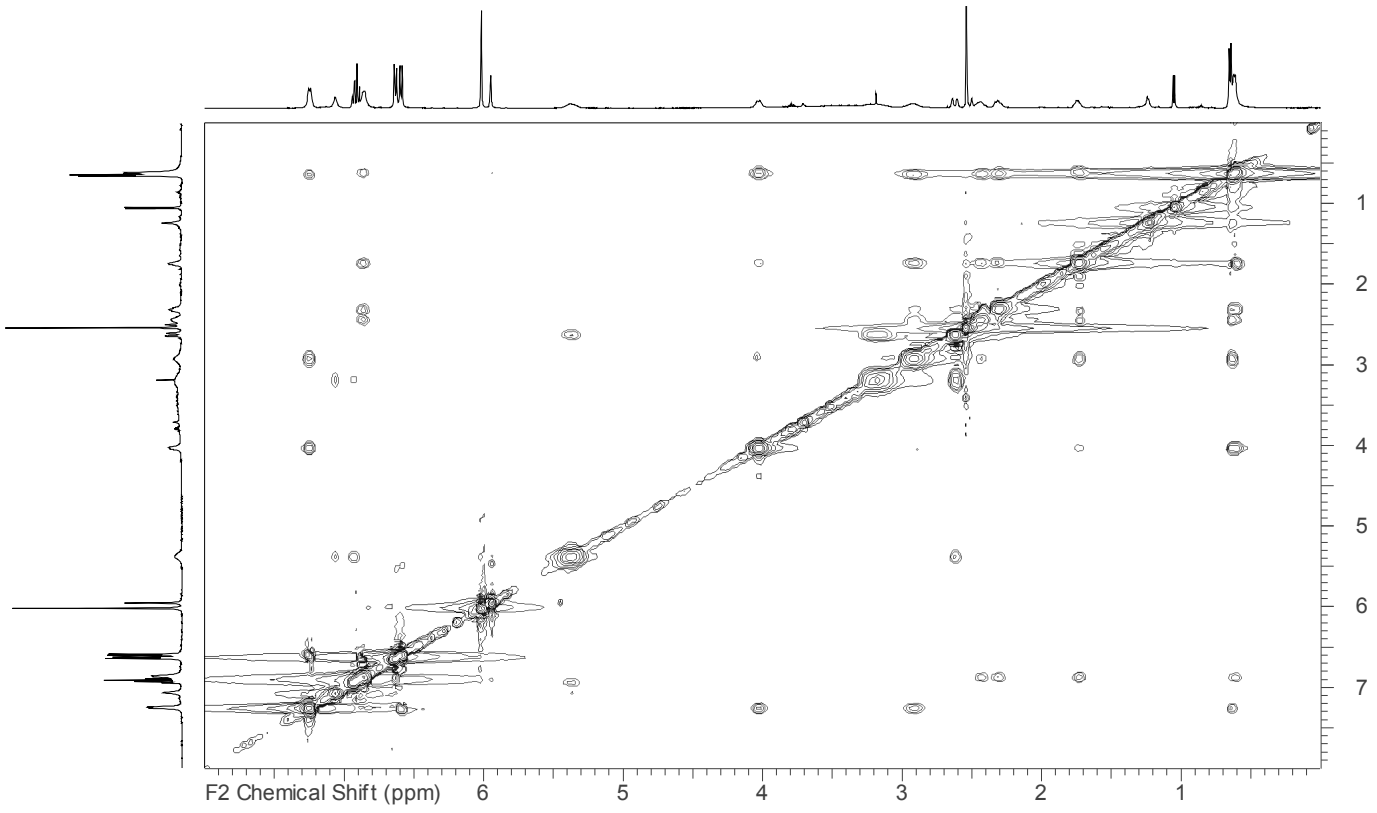

Figure S47. ^{1}H-^{1}H ROESY spectrum of compound **10** (500 MHz, DMSO-d_6)

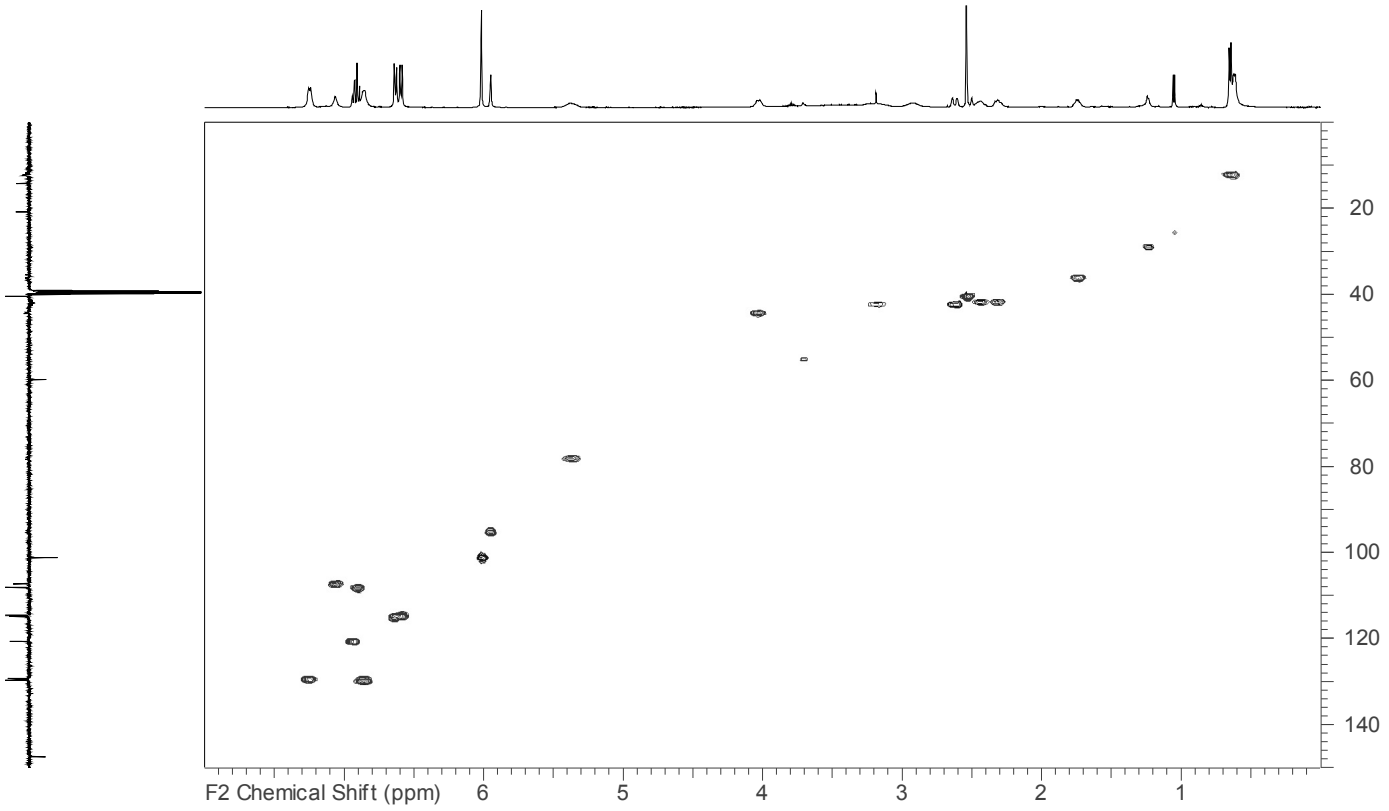

Figure S48. HSQC-DEPT spectrum of compound **10** (500 MHz, DMSO-d_6)

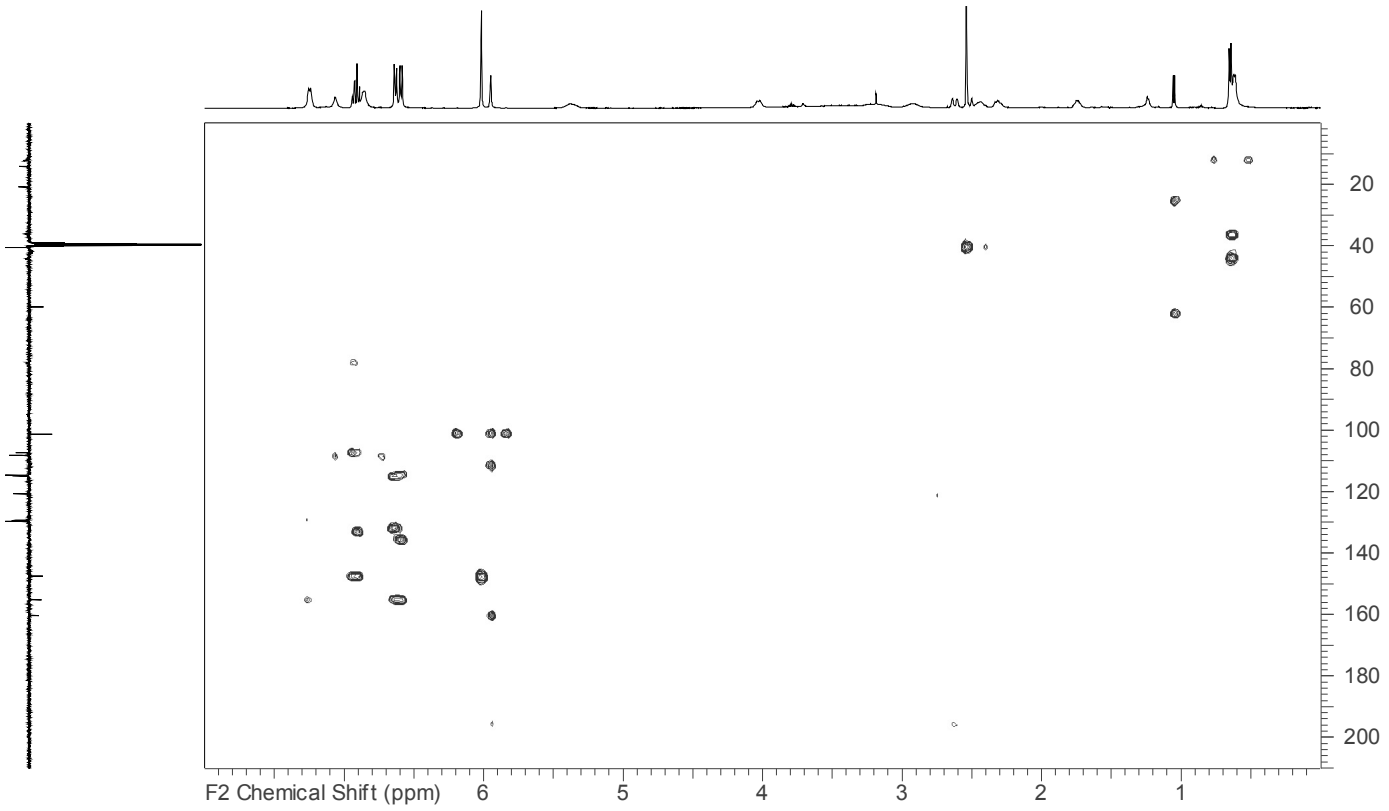

Figure S49. HMBC spectrum of compound **10** (500 MHz, DMSO-d_6)

4. CONCLUSION AND OUTLOOK

There is an urgent need to find safer substitutes for copper pesticides in organic agriculture. In this context, an in-house library of plant and fungal extracts was screened *in vitro* against *Plasmopara viticola* (grapevine downy mildew), *Phytophthora infestans* (potato and tomato late blight), and *Venturia inaequalis* (apple scab). As a result of this screening, several plants were selected for further investigation in the course of this thesis, namely *Juncus effusus* (Juncaceae), *Styrax tonkinensis* and *S. paralleloneurum*/*benzoin* (Styracaceae), *Magnolia officinalis* (Magnoliaceae), *Verbesina lanata* (Asteraceae), and *Iryanthera megistocarpa* (Myristicaceae).

The ethyl acetate extract of *J. effusus* medulla (Chapter 3.1) showed promising efficacies on grapevine seedlings against *P. viticola* and on apple seedlings against *V. inaequalis*. The main active compound was identified as dehydroeffusol, a dehydrophenanthrene. *J. effusus* is almost cosmopolitan and its medulla is used in Traditional Chinese Medicine. Consequently, it is easily available at reasonable prices. In addition, phenanthrenes are lipophilic compounds, which make them advantageous in terms of rain-fastness. However, the low density of the medulla which would necessitate large storage rooms and the low extraction yield (0.5%) may represent shortcomings for further development. In organic farming, extracts or compounds directly isolated from the plant material should be used, since no synthetic products are allowed. Therefore, synthesis of the active constituents would not be an alternative approach. In addition, some phenanthrene derivatives have been reported to show toxicity towards several trophic levels of the ecosystem. Consequently, specific ecotoxicological studies will have to be performed to ensure product safety.

Siam benzoin (SB), the resin from *Styrax tonkinensis*, and Sumatra benzoin (SumB), the resin from *Styrax benzoin* or *Styrax paralleloneurum* (Chapter 3.2) were highly active *in vitro* against the three pathogens. The active compounds were identified to be coniferyl benzoate (SB) and *p*-coumaryl cinnamate (SumB). On grapevine seedlings against *P. viticola* and on apple seedlings against *V. inaequalis*, the two resins and pure compounds showed excellent efficacies. In addition, SB showed outstanding efficacies on tomato seedlings against *P. infestans*. On the seedlings, these products were even more efficient than copper under semi-controlled conditions. SB was also active against the fungus *Diplocarpon mali* Y. Harada & Sawamura (anamorph *Marssonina coronaria*) causing Marssonina blotch of apple [335]. Under field conditions on grapevine, SB showed significant reduction of the infestation of *P. viticola* (downy mildew) and *Uncinula necator*, anamorph *Oidium tuckeri* (powdery mildew)

[54] in two different years (2014 and 2015). SumB is currently under evaluation in field conditions on grapevine and apple trees. An European Patent Application has been filed in order to further develop these natural products. Since SB and SumB are part of many Pharmacopoeia, used since ancient times in traditional medicine, and widely utilised in agro-alimentary industries, they are considered as safe for humans. As a result, SumB and SB are available in sufficient quantities for field application. Furthermore, the resins are fully soluble in ethanol and the concentration of the active compounds is high (up to 53% in SB and up to 29% in SumB). As a next step, in-depth toxicity assessments will have to be performed to ensure safety on the environment. Further product formulations should be tested to possibly improve the activity under field condition. Finally, field testing could be extended to further crops.

The ethyl acetate extract of *M. officinalis* bark (Chapter 3.3) showed high *in vitro* activity against the three pathogens. The active compounds were identified as the two neolignans, magnolol and honokiol. On grapevine and apple seedlings, the efficacies were similar to that of copper, while on tomato seedlings (*P. infestans*), the efficacy was significantly lower. On field trials, the activity on apple scab could not be confirmed. In the year 2014, the efficacy against *P. viticola* on vineyards was low after application of the raw extract, probably due to solubility issues. The efficacies were considerably increased by the development of formulations and the performance of a wettable powder was comparable to the reference organic fungicide. *M. officinalis* bark has a potential for further development as a plant protection product. The bark of *M. officinalis* is widely used in Traditional Chinese Medicine, in cosmetics, and in food supplements. Thus, the drug is available in large amounts at reasonable prices and is considered as safe for humans. In addition, the extraction yield of approximately 7% is acceptable and magnolol/honokiol represent up to 30% of the extract. However, toxicity reports on magnolol showed effects on different trophic levels. Consequently, to guarantee product safety, further ecotoxicological studies have to be performed.

An ethyl acetate extract of *V. lanata* leaves (Chapter 3.4) showed promising *in vitro* activity against *P. viticola*. The efficacy on grapevine seedlings was initially low, which was probably due to solubility issues of the extract. Therefore, a preliminary formulation was developed in the form of an emulsifiable concentrate to enhance solubility in water. With this formulation, at a concentration of 1 mg/mL of extract, the leaf surface infestation was

reduced by 82% compared to the non-treated control. From the ethyl acetate extract, 16 eudesmane sesquiterpenes with a cinnamoyloxy group corresponding to the major UV-peaks of the chromatogram were isolated. Among them, eight were new congeners. Their relative configuration was assigned by NOESY correlations and coupling constants, while their absolute configuration was established by ECD measurements in combination with quantum calculations. Nine of these compounds were obtained in sufficient amounts to be tested *in vitro* against *P. viticola*, where five of them showed MIC_{100} values lower than 10 μg/mL.. Toxicological evaluation of this compound class and of the crude extract should be carried out to assess safety and tests under field conditions should be performed. Moreover, in view of the low extraction yield (approx. 2.5% with the current procedure) and limited access to the plant material, cultivation of *V. lanata* would be required. The use of other plants containing this type of eudesmane sesquiterpenes in larger quantities may also be an alternative.

The ethyl acetate of *I. megistocarpa* leaves (Chapter 3.5) exhibited good activity *in vitro* against the three pathogens. On grapevine and tomato seedlings, at a concentration of 1 mg/mL, it reduced leaf infestation by 87% and 68%, respectively. Tests on apple seedlings are ongoing. Two dihydrochalcones and eight flavonolignans including several stereoisomers were isolated from the extract with the two most active compounds showing MIC_{100} values $\leq$2.3 μg/mL against each tested pathogen. A broad variation was observed for some pure compounds between independent *in vitro* assays. No precipitation was observed after compound addition into the test wells and no such variation was observed for controls or compounds from other sources tested in parallel. Additional investigations will be needed to identify the causes of these unexpected potency variations. Furthermore, the highly limited access to the plant (growing only in geographically restricted areas of Panama) would necessitate the development of cultivation, since these types of flavonolignans are reportedly specific to *Iryanthera* species. In addition, as for the other investigated plants, some toxicological studies should be performed. Finally, stereochemical assignment is still needed for the flavonolignans **3**-**6**, **9**, and **10**, which possess three different planar structures with several configurations. Possible free rotation along the side chain makes such a determination particularly challenging. Further crystallisation attempts, possibly after the preparation of appropriate derivatives, should be made.

In this thesis, we could confirm that plant-derived products have a potential for the development of new natural pesticides which could replace, or at least reduce the use of copper. Natural products possess several advantages compared to synthetic pesticides, such as being safer for the environment due to their rapid biodegradation, showing mainly low toxicity to non-target organisms and humans, or preventing the development of resistance [41, 50]. However, there are some inherent difficulties: (i) the efficacy is often limited by the short compound persistence on the field, (ii) the quantity needed to reach an appreciable efficacy is high, (iii) the availability and sustainable supply of plant material is challenging which leads to high production costs, and (iiii) regulatory barriers for the development of new products [20, 41, 43, 49, 50] may make a development economically not attractive. Nevertheless, the interest for pesticides from natural origins is expected to raise in the upcoming years since the World Health Organization strongly supports their use and the public is becoming increasingly aware of food safety and ecological issues [50]. Considering this, the successful cooperation between the Pharmaceutical Biology Group of the University of Basel and the Forschungsinstitut für biologischen Landbau (FiBL) is continuing and several further promising plant extracts are now under investigation in the context of a following PhD project.

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REFERENCES

1. Matson, P.A., Parton, W.J., Power, A.G., and Swift, M.J., *Agricultural Intensification and Ecosystem Properties.* Science, 1997. **277**(5325): p. 504-509.
2. Wilson, C. and Tisdell, C., *Why Farmers Continue to Use Pesticides despite Environmental, Health and Sustainability Costs.* Ecological Economics, 2001. **39**(3): p. 449-462.
3. Tamm, L., Pertot, I., and Gubler, W.D., *Chapter 5.7: Organic Grape Disease Management*, in *Plant Diseases and Their Management in Organic Agriculture*, M.R. Finckh, A.H.C. van Bruggen, and L. Tamm, Editors. 2015, The American Phytopathological Society: St. Paul. p. 335-350.
4. Dagostin, S., Schärer, H.-J., Pertot, I., and Tamm, L., *Are There Alternatives to Copper for Controlling Grapevine Downy Mildew in Organic Viticulture?* Crop Protection, 2011. **30**(7): p. 776-788.
5. Kühne, S., Rossberg, D., Röhrig, P., von Mering, F., Weihrauch, F., Kanthak, S., Kienzle, J., Patzwahl, W., and Reiners, E., *Status Quo der Anwendung kupferhaltiger Pflanzenschutzmittel in der deutschen Landwirtschaft und dem Gartebau.* Journal für Kultupflanzen, 2016. **68**(7): p. 189-196.
6. Flemming, C.A. and Trevors, J.T., *Copper Toxicity and Chemistry in the Environment: A Review.* Water, Air, and Soil Pollution, 1989. **44**(1-2): p. 143-158.
7. Potterat, O. and Hamburger, M., *Combined Use of Extract Libraries and HPLC-Based Activity Profiling for Lead Discovery: Potential, Challenges, and Practical Considerations.* Planta Medica, 2014. **80**(14): p. 1171-1181.
8. *World Population Prospects: The 2017 Revision*, 2017, Department of Economic and Social Affairs, United Nations.
9. Godfray, H.C.J., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M., and Toulmin, C., *Food Security: The Challenge of Feeding 9 Billion People.* Science, 2010. **327**(5967): p. 812-818.
10. Unsworth, J.B., Corsi, C., Van Emon, J.M., Farenhorst, A., Hamilton, D.J., Howard, C.J., Hunter, R., Jenkins, J.J., Kleter, G.A., Kookana, R.S., Lalah, J.O., Legget, M., Miglioranza, K.S.B., Miyagawa, H., Peranginangin, N., Rubin, B., Saha, B., and Shakil, N.A., *Developing Global Leaders for Research, Regulation, and Stewardship of Crop Protection Chemistry in the 21st Century.* Journal of Agricultural and Food Chemistry, 2016. **64**(1): p. 52-60.
11. Foley, J.A., Ramankutty, N., Brauman, K.A., Cassidy, E.S., Gerber, J.S., Johnston, M., Mueller, N.D., O'Connell, C., Ray, D.K., West, P.C., Balzer, C., Bennett, E.M., Carpenter, S.R., Hill, J., Monfreda, C., Polasky, S., Rockström, J., Sheehan, J., Siebert, S., Tilman, D., and Zaks, D.P.M., *Solutions for a Cultivated Planet.* Nature, 2011. **478**(7369): p. 337-342.
12. Ntalli, N.G. and Menkissoglu-Spiroudi, U., *Chapter 1: Pesticides of Botanical Origin: A Promising Tool in Plant Protection*, in *Pesticides - Formulations, Effects, Fate*, M. Stoytcheva, Editor. 2011, InTech: Rijeka. p. 3-24.

13. Connor, D.J. and Minguez, M.I., *Evolution not Revolution of Farming Systems Will Best Feed and Green the World.* Global Food Security, 2012. **1**(2): p. 106-113.

14. Dang, Q.L., Lim, C.H., and Kim, J.-C., *Current Status of Botanical Pesticides for Crop Protection.* Research in Plant Disease, 2012. **18**(3): p. 175-185.

15. Bolognesi, C., *Genotoxicity of Pesticides: A Review of Human Biomonitoring Studies.* Mutation Research-Reviews in Mutation Research, 2003. **543**(3): p. 251-272.

16. Dayan, F.E., Cantrell, C.L., and Duke, S.O., *Natural Products in Crop Protection.* Bioorganic & Medicinal Chemistry, 2009. **17**(12): p. 4022-4034.

17. Lorenz, E.S., *Pesticide Safety Fact Sheet: Potential Health Effects of Pesticides.* PennState Extension, 2009: p. 1-8.

18. Lechenet, M., Dessaint, F., Py, G., Makowski, D., and Munier-Jolain, N., *Reducing Pesticide Use while Preserving Crop Productivity and Profitability on Arable Farms.* Nature Plants, 2017. **3**(17008): p. 1-6.

19. Vasileiadis, V.P., *Economic Sustainability: Less Pesticide Rarely Causes Loss.* Nature Plants, 2017. **3**(17016): p. 1-2.

20. El-Wakeil, N.E., *Botanical Pesticides and Their Mode of Action.* Gesunde Pflanzen, 2013. **65**(4): p. 125-149.

21. Pimentel, D., *Environmental and Economic Costs of the Application of Pesticides Primarily in the United States.* Environment, Development and Sustainability, 2005. **7**(2): p. 229-252.

22. Weisenburger, D.D., *Human Health Effects of Agrichemical Use.* Human Pathology, 1993. **24**(6): p. 571-576.

23. Badach, H., Nazimek, T., Kaminski, R., and Turski, W.A., *Organochlorine Pesticides Concentration in the Drinking Water from Regions of Extensive Agriculture in Poland.* Annals of Agricultural and Environmental Medicine, 2000. **7**(1): p. 25-28.

24. Gilliom, R.J., *Pesticides in US Streams and Groundwater.* Environmental Science and Technology, 2007. **41**(10): p. 3408-3414.

25. Mullin, C.A., Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., vanEngelsdorp, D., and Pettis, J.S., *High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health.* PLoS One, 2010. **5**(3): p. e9754.

26. Schwarzenbach, R.P., Egli, T., Hofstetter, T.B., von Gunten, U., and Wehrli, B., *Global Water Pollution and Human Health.* Annual Review of Environment and Resources, 2010. **35**: p. 109-136.

27. Maloy, O.C., *Plant Disease Management.* The Plant Health Instructor, 2005: p. DOI: 10.1094/PHI-I-2005-0202-01.

28. McGrath, M.T., *What are fungicides?* The Plant Health Instructor, 2004: p. DOI: 10.1094/PHI-I-2004-0825-01.

29. Cressey, D., *The Bitter Battle over the World's Most Popular Insecticides.* Nature News, 2017. **551**(7679): p. 156-158.

30. Tilman, D., *Global Environmental Impacts of Agricultural expansion: The Need for Sustainable and Efficient Practices.* Proceedings of the National Academy of Sciences, 1999. **96**(11): p. 5995-6000.

31. Philogene, B.J.R., Regnault-Roger, C., and Vincent, C., *Chapitre 1: Biopesticides d'Origine Végétale: Bilan et Perspectives*, in *Biopesticides d'Origine Végétale*, C. Regnault-Roger, B.J.R. Philogene, and C. Vincent, Editors. 2008, Lavoisier Tec & Doc: Paris. p. 1-24.

32. Bourguet, D. and Guillemaud, T., *Chapter 2: The Hidden and External Costs of Pesticide Use*, in *Sustainable Agriculture Reviews: Volume 19*, E. Lichtfouse, Editor. 2016, Springer International Publishing Switzerland: Cham. p. 35-120.

33. Pretty, J.N., Brett, C., Gee, D., Hine, R.E., Mason, C.F., Morison, J.I.L., Raven, H., Rayment, M.D., and van der Bijl, G., *An Assessment of the Total External Costs of UK Agriculture.* Agricultural Systems, 2000. **65**(2): p. 113-136.

34. Barkai-Golan, R., *Chapter 9: Chemical Control*, in *Postharvest Diseases of Fruits and Vegetables*. 2001, Elsevier: Amsterdam. p. 147-188.

35. Gonzalez-Coloma, A., Reina, M., Diaz, C.E., Fraga, B.M., and Santana-Meridas, O., *Natural Product-Based Biopesticides for Insect Control*, in *Elsevier Reference Module in Chemistry, Molecular Sciences and Chemical Engineering*, J. Reedijk, Editor. 2013, Elsevier: Waltham.

36. Duke, S.O., Cantrell, C.L., Meepagala, K.M., Wedge, D.E., Tabanca, N., and Schrader, K.K., *Natural Toxins for Use in Pest Management.* Toxins, 2010. **2**(8): p. 1943-1962.

37. Wedge, D.E. and Camper, N.D., *Chapter 1: Connections between Agrochemicals and Pharmaceuticals*, in *Biologically Active Natural Products: Pharmaceuticals*, S.J. Cutler and H.G. Cutler, Editors. 2000, CRC Press LLC: Boca Raton. p. 1-15.

38. Castillo, F., Hernandez, D., Gallegos, G., Rodriguez, R., and Aguilar, C.N., *Chapter 4: Antifungal Properties of Bioactive Compounds from Plants*, in *Fungicides for Plant and Animal Diseases*, D. Dhanasekaran, N. Thajuddin, and A. Panneerselvam, Editors. 2011, InTech: Rijeka. p. 91-106.

39. Seiber, J.N., Coats, J., Duke, S.O., and Gross, A.D., *Biopesticides: State of the Art and Future Opportunities.* Journal of Agricultural and Food Chemistry, 2014. **62**(48): p. 11613-11619.

40. Rimando, A.M. and Duke, S.O., *Chapter 1: Natural Products for Pest Management*, in *Natural Products for Pest Management*, A.M. Rimando and S.O. Duke, Editors. 2006, American Chemical Society: Washington DC. p. 2-21.

41. Pavela, R., *Chapter 17: Limitation of Plant Biopesticides*, in *Advances in Plant Biopesticides*, D. Singh, Editor. 2014, Springer India: New Delhi. p. 347-359.

42. Loiseleur, O., *Natural Products in the Discovery of Agrochemicals.* CHIMIA International Journal for Chemistry, 2017. **71**(12): p. 810-822.

43. Guleria, S. and Tiku, A.K., *Chapter 12: Botanicals in Pest Management: Current Status and Future Perspectives*, in *Integrated Pest Management: Innovation-Development Process: Volume 1*, R. Peshin and A.K. Dhawan, Editors. 2009, Springer Netherlands: Dordrecht. p. 317-329.

44. Chiasson, H., Delisle, U., Bostanian, N.J., and Vincent, C., *Chapitre 22: Recherche, Développement et Commercialisation de Facin™, un Biopesticide d'Origine Végétale. Etude d'un Cas de Réussite en Amérique du Nord*, in *Biopesticides d'Origine Végétale*, C. Regnault-Roger, B.J.R. Philogene, and C. Vincent, Editors. 2008, Lavoisier Tec & Doc: Paris. p. 452-463.

45. Copping, L.G. and Menn, J.J., *Biopesticides: A Review of their Action, Applications and Efficacy.* Pest Management Science, 2000. **56**(8): p. 651-676.

46. Walia, S., Saha, S., and Rana, V.S., *Chapter 15: Phytochemical Pesticides*, in *Advances in Plant Biopesticides*, D. Singh, Editor. 2014, Springer India: New Delhi. p. 295-322.

47. Verpoorte, R., *Exploration of Nature's Chemodiversity: The Role of Secondary Metabolites as Leads in Drug Development.* Drug Discovery Today, 1998. **3**(5): p. 232-238.

48. Martinez, J.A., *Chaper 1: Natural Fungicides Obtained from Plants*, in *Fungicides for Plant and Animal Diseases*, D. Dhanasekaran, N. Thajuddin, and A. Panneerselvam, Editors. 2011, InTech: Rijeka. p. 3-28.

49. Isman, M.B., *Botanical Insecticides, Deterrents, and Repellents in Modern Agriculture and an Increasingly Regulated World.* Annual Review of Entomology, 2006. **51**(1): p. 45-66.

50. Bhagat, S., Birah, A., Kumar, R., Yadav, M.S., and Chattopadhyay, C., *Chapter 7: Plant Disease Management: Prospects of Pesticides of Plant Origin*, in *Advances in Plant Biopesticides*, D. Singh, Editor. 2014, Springer India: New Delhi. p. 119-129.

51. Hüter, O.F., *Use of Natural Products in the Crop Protection Industry.* Phytochemistry Reviews, 2011. **10**(2): p. 185-194.

52. Isman, M.B., *Chapitre 23: Problèmes et Perspectives de Commercialisation des Insecticides d'Origine Botanique*, in *Biopesticides d'Origine Végétale*, C. Regnault-Roger, B.J.R. Philogene, and C. Vincent, Editors. 2008, Lavoisier Tec & Doc: Paris. p. 466-476.

53. Paranjape, K., Gowariker, V., Krishnamurthy, V.N., and Gowariker, S., *The Pesticide Encyclopedia* 2014, Wallingford - Boston: CABI.

54. Agrios, G.N., *Plant Pathology*. Fourth ed 1997, San Diego: Academic Press.

55. Schmale III, D.G. and Munkvold, G.P., *Mycotoxins in Crops: A Threat to Human and Domestic Animal Health.* The Plant Health Instructor, 2009: p. DOI: 10.1094/PHI-I-2009-0715-01 Updated 2014.

56. Knight, S.C., Anthony, V.M., Brady, A.M., Greenland, A.J., Heaney, S.P., Murray, D.C., Powell, K.A., Schulz, M.A., Spinks, C.A., Worthington, P.A., and Youle, D., *Rationale and Perspectives on the Development of Fungicides.* Annual Review of Phytopathology, 1997. **35**: p. 349-372.

57. Krska, R. and Crews, C., *Significance, Chemistry and Determination of Ergot Alkaloids: A Review.* Food Additives and Contaminants, 2008. **25**(6): p. 722-731.

58. *Common and Trade Funigicides.* APS Education Center, 2016.

59. Gerwick, B.C. and Sparks, T.C., *Natural Products for Pest Control: An Analysis of their Role, Value and Future.* Pest Management Science, 2014. **70**(8): p. 1169-1185.

60. Sparks, T.C., Hahn, D.R., and Garizi, N.V., *Natural Products, their Derivatives, Mimics and Synthetic Equivalents: Role in Agrochemical Discovery.* Pest Management Science, 2017. **73**(4): p. 700-715.

61. Hofmann, U., *Kupferreduzierung und Kupferersatz im ökologischen Weinbau - Ergebnisse aus dem BÖW - Ringversuch*, in *Alternativen zur Anwendung von Kupfer als Pflanzschutzmittel. 7. Fachgespräch am 6. Juni 2002 in Berlin-Dahlem. Berichte aus der biologischen Bundesanstalt für Land- und Forstwirtschaft*2003. p. 27-37.

62. *Plant Extract 620 (169007) Fact Sheet*, United States Environmental Protection Agency (EPA).

63. Cantrell, C.L., Dayan, F.E., and Duke, S.O., *Natural Products as Sources for New Pesticides.* Journal of Natural Products, 2012. **75**(6): p. 1231-1242.

64. *Oriental Mustard Seed; Allyl isothiocyanate (AITC) (014921) Fact Sheet*, United States Environmental Protection Agency (EPA).

65. Stuardo, M. and San Martín, R., *Antifungal Properties of Quinoa (*Chenopodium quinoa *Willd) Alkali Treated Saponins against Botrytis cinerea.* Industrial Crops and Products, 2008. **27**(3): p. 296-302.

66. *Saponins of* Chenopodium quinoa *(097094) Fact Sheet*, United States Environmental Protection Agency (EPA).

67. *Saponins of* Quillaja saponaria *(097095) Fact Sheet*, United States Environmental Protection Agency (EPA).

68. Tamm, L., Speiser, B., and Holb, I., *Chapter 4.7: Direct Control of Airborne Diseases*, in *Plant Diseases and Their Management in Organic Agriculture*, M.R. Finckh, A.H.C. van Bruggen, and L. Tamm, Editors. 2015, The American Phytopathological Society (APS): St. Paul. p. 205-216.

69. Copping, L.G. and Duke, S.O., *Natural Products That Have Been Used Commercially as Crop Protection Agents.* Pest Management Science, 2007. **63**(6): p. 524-554.

70. Yoon, M.-Y., Cha, B., and Kim, J.-C., *Recent Trends in Studies on Botanical Fungicides in Agriculture.* The Plant Pathology Journal, 2013. **29**(1): p. 1-9.

71. Dewick, P.M., *Medicinal Natural Products: A Biosynthetic Approach*. Third ed 2011, Chichester: Wiley.

72. Bang, K.-H., Lee, D.-W., Park, H.-M., and Rhee, Y.-H., *Inhibition of Fungal Cell Wall Synthesizing Enzymes by* trans-*Cinnamaldehyde.* Bioscience, Biotechnology, and Biochemistry, 2000. **64**(5): p. 1061-1063.

73. Pajot, E. and Regnault-Roger, C., *Chapitre 11: Stimulation des Défenses Naturelles des Plantes et Résistance Induite: Une Nouvelle Approche Phytosanitaire?*, in *Biopesticides d'Origine Végétale*, C. Regnault-Roger, B.J.R. Philogene, and C. Vincent, Editors. 2008, Lavoisier Tec & Doc: Paris. p. 231-257.

74. Konstantinidou-Doltsinis, S., Markellou, E., Kasselaki, A.-M., Fanouraki, M.N., Koumaki, C.M., Schmitt, A., Liopa-Tsakalidis, A., and Malathrakis, N.E., *Efficacy of*

Milsana®, a Formulated Plant Extract from Reynoutria sachalinensis*, against Powdery Mildew of Tomato (*Leveillula taurica*).* BioControl, 2006. **51**(3): p. 375-392.

75. Daayf, F., Schmitt, A., and Belanger, R.R., *Evidence of Phytoalexins in Cucumber Leaves Infected with Powdery Mildew following Treatment with Leaf Extracts of* Reynoutria sachalinensis*.* Plant Physiology, 1997. **113**(3): p. 719-727.

76. Trottin-Caudal, Y., Fournier, C., Leyre, J.M., Decognet, V., Romiti, C., Nicot, P., and Bardin, M., *Efficiency of Plant Extract from* Reynoutria sachalinensis *(Milsana) to Control Powdery Mildew on Tomato (*Oidium neolycopersici*)*, in *Colloque International Tomate sous Abri, Protection Intégrée - Agriculture Biologique, Avignon, France, 17-18 et 19 Septembre 2003*, L. Roche, et al., Editors. 2003, Centre Technique Interprofessionnel des Fruits et Légumes: Paris. p. 11-15.

77. Daayf, F., Schmitt, A., and Belanger, R.R., *The Effects of Plant Extracts of* Reynoutria sachalinensis *on Powdery Mildew Development and Leaf Physiology of Long English Cucumber.* Plant Disease, 1995. **79**(6): p. 577-580.

78. Benhamou, N. and Rey, P., *Stimulateurs des Défenses Naturelles des Plantes: Une Nouvelle Stratégie Phytosanitaire dans un Contexte d'Ecoproduction Durable. II. Intérêt des SDN en Protection des Cultures.* Phytoprotection, 2012. **92**(1): p. 24-35.

79. Delves-Broughton, J., *Permitted Preservatives - Natamycin*, in *Encyclopedia of Food Microbiology*, R.K. Robinson and M.L. Tortorello, Editors. 2014, Elsevier: Oxford. p. 87-91.

80. Irfan-Maqsood, M. and Seddiq-Shams, M., *Rhamnolipids: Well-Characterized Glycolipids with Potential Broad Applicability as Biosurfactants.* Industrial Biotechnology, 2014. **10**(4): p. 285-291.

81. Bartlett, D.W., Clough, J.M., Godwin, J.R., Hall, A.A., Hamer, M., and Parr-Dobrzanski, B., *The Strobilurin Fungicides.* Pest Management Science, 2002. **58**(7): p. 649-662.

82. Vincelli, P., *Q_o1 (Strobilurin) Fungicides: Benefits and Risks.* The Plant Health Instructor, 2002: p. DOI: 10.1094/PHI-I-2002-0809-02. Updated, 2012.

83. Gessler, C., Pertot, I., and Perazzolli, M., Plasmopara viticola*: A Review of Knowledge on Downy Mildew of Grapevine and Effective Disease Management.* Phytopathologia Mediterranea, 2011. **50**: p. 3-44.

84. Hajji, S., Younes, I., Ghorbel-Bellaaj, O., Hajji, R., Rinaudo, M., Nasri, M., and Jellouli, K., *Structural Differences Between Chitin and Chitosan Extracted from Three Different Marine Sources.* International Journal of Biological Macromolecules, 2014. **65**: p. 298-306.

85. El Hadrami, A., Adam, L.R., El Hadrami, I., and Daayf, F., *Chitosan in Plant Protection.* Marine Drugs, 2010. **8**(4): p. 968-987.

86. Bradshaw, C.J.A., Leroy, B., Bellard, C., Roiz, D., Albert, C., Fournier, A., Barbet-Massin, M., Salles, J.-M., Simard, F., and Courchamp, F., *Massive yet Brossly Underestimated Global Costs of Invasive Insects.* Nature Communications, 2016. **7**(12986): p. 1-8.

87. Weber, D., *Colorado Beetle: Pest on the Move.* Pesticide Outlook, 2003. **14**(6): p. 256-259.

88. Gale, G., *Chapter 4: Saving the Vine from Phylloxera: A Never Ending Battle*, in *Wine: A scientific exploration*, M. Sandler and R. Pinder, Editors. 2003, Taylor & Francis: London. p. 70-91.

89. Guiser, S., *Organic Insecticides.* PennState Extension, 2017: p. 1-3.

90. *Extract of* Chenopodium ambrosioides *near* ambrosioides *(599995) Fact Sheet*, United States Environmental Protection Agency (EPA).

91. Reiter, S. *REQUIEM: A New Product for Control of Thrips, Whiteflies, Mites and Other Sucking Pests* in *4th Annual Biocontrol Industry Meeting (ABIM)*. 19-20th October 2009. Lucerne.

92. *Pesticides and Alternatives.* PennState Extension, 2017: p. 1-7.

93. Schmutterer, H., *Properties and Potential of Natural Pesticides from the Neem Tree,* Azadirachta indica*.* Annual Review of Entomology, 1990. **35**(1): p. 271-297.

94. Salehzadeh, A., Akhkha, A., Cushley, W., Adams, R.L.P., Kusel, J.R., and Strang, R.H.C., *The Antimitotic Effect of the Neem Terpenoid Azadirachtin on Cultured Insect Cells.* Insect Biochemistry and Molecular Biology, 2003. **33**(7): p. 681-689.

95. Ware, G.W., *The Pesticide Book*. Fifth ed 2000, Fresno: Thomson Publications.

96. Shivanandappa, T. and Rajashekar, Y., *Chapter 16: Mode of Action of Plant-Derived Natural Insecticides*, in *Advances in Plant Biopesticides*, D. Singh, Editor. 2014, Springer India: New Delhi. p. 323-345.

97. Isman, M.B. and Akhtar, Y., *Chapter 10: Plant Natural Products as a Source for Developing Environmentally Acceptable Insecticides*, in *Insecticides design using advanced technologies*, I. Ishaaia, R. Nauen, and A.R. Horowitz, Editors. 2007, Springer-Verlag: Berlin-Heidelberg. p. 235-248.

98. Dimetry, N.Z., *Chapter 1: Different Plant Families as Bioresource for Pesticides*, in *Advances in Plant Biopesticides*, D. Singh, Editor. 2014, Springer India: New Delhi. p. 1-20.

99. Casida, J.E., Pyrethrum *Flowers and Pyrethroid Insecticides.* Environmental Health Perspectives, 1980. **34**: p. 189-202.

100. *Pesticide Information Profile: Abamectin.* EXTOXNET: The Extension Toxicology Network, 1994.

101. Lasota, J.A. and Dybas, R.A., *Abamectin as a Pesticide for Agricultural Use.* Acta Leidensia, 1990. **59**(1-2): p. 217-225.

102. Tomizawa, M. and Casida, J.E., *Neonicotinoids Insecticide Toxicology: Mechanisms of Selective Action.* Annual Review of Pharmacology and Toxicology, 2005. **45**: p. 247-268.

103. Sparks, T.C., Crouse, G.D., and Durst, G., *Natural Products as Insecticides: The Biology, Biochemistry and Quantitative Structure–Activity Relationships of Spinosyns and Spinosoids.* Pest Management Science, 2001. **57**(10): p. 896-905.

104. Galm, U. and Sparks, T.C., *Natural Product Derived Insecticides: Discovery and Development of Spinetoram.* Journal of Industrial Microbiology & Biotechnology, 2016. **43**(2): p. 185-193.

105. Kim, Y.K., Tomoda, H., Nishida, H., Sunazuka, T., Obata, R., and Omura, S., *Pyripyropenes, Novel Inhibitors of Acyl-CoA: Cholesterol Acyltransferase Produced by* Aspergillus fumigatus */ II. Structure Elucidation of Pyripyropene A, B, C and D.* . The Journal of Antibiotics, 1994. **47**(2): p. 154-162.

106. Kandasamy, R., London, D., Stam, L., von Deyn, W., Zhao, X., Salgado, V.L., and Nesterov, A., *Afidopyropen: New and Potent Modulator of Insect Transient Receptor Potential Channels.* Insect Biochemistry and Molecular Biology, 2017. **84**: p. 32-39.

107. Khambay, B.P.S. and Jewess, P., *The Potential of Natural Naphthoquinones as the Basis for a New Class of Pest Control Agents — An Overview of Research at IACR-Rothamsted.* Crop Protection, 2000. **19**(8): p. 597-601.

108. Dekeyser, M.A., *Review: Acaricide Mode of Action.* Pest Management Science, 2005. **61**(2): p. 103-110.

109. Addor, R.W., Babcock, T.J., Black, B.C., Brown, D.G., Diehl, R.E., Furch, J.A., Kameswaran, V., Kamhi, V.M., Kremer, K.A., Kuhn, D.G., Lovell, J.B., Lowen, G.T., Miller, T.P., Peevey, R.M., Siddens, J.K., Treacy, M.F., Trotto, S.H., and Wright, D.P., *Chapter 25: Insecticidal Pyrroles, Discovery and Overview*, in *Synthesis and Chemistry of Agrochemicals III*, D.R. Baker, J.G. Fenyes, and J.J. Steffens, Editors. 1992, American Chemical Society: Washington DC. p. 283-297.

110. Charan, R.D., Schlingmann, G., Bernan, V.S., Feng, X., and Carter, G.T., *Dioxapyrrolomycin Biosynthesis in* Streptomyces fumanus. Journal of Natural Products, 2006. **69**(1): p. 29-33.

111. Black, B.C., Hollingworth, R.M., Ahammadsahib, K.I., Kukel, C.D., and Donovan, S., *Insecticidal Action and Mitochondrial Uncoupling Activity of AC-303,630 and Related Halogenated Pyrroles.* Pesticide Biochemistry and Physiology, 1994. **50**(2): p. 115-128.

112. *Chlorfenapyr (129093) Pesticide Fact Sheet*, 2001, United States Environmental Protection Agency (EPA).

113. King, G.F. and Hardy, M.C., *Spider-Venom Peptides: Structure, Pharmacology, and Potential for Control of Insect Pests.* Annual Review of Entomology, 2013. **58**: p. 475-496.

114. Isbister, G.K. and Fan, H.W., *Spider Bite.* The Lancet, 2011. **378**(9808): p. 2039-2047.

115. *Vestaron SPEAR™ Products Mechanisms of Action*, 2016, Vestaron, Nature by Design.

116. Graf, J., *Shifting Paradigm on* Bacillus thuringiensis *Toxin and a Natural Model for* Enterococcus faecalis *Septicemia.* mBio, 2011. **2**(4): p. 1-2.

117. Peralta, C. and Palma, L., *Is the Insect World Overcoming the Efficacy of* Bacillus thuringiensis*?* Toxins, 2017. **9**(1): p. 39-43.

118. Lingenfelter, D.D. and Hartwig, N.L., *Introduction to Weeds and Herbicides.* PennState Extension, 2013: p. 1-28.

119. Dayan, F.E. and Duke, S.O., *Natural Compounds as Next-Generation Herbicides.* Plant Physiology, 2014. **166**(3): p. 1090-1105.

120. Lee, D.L., Prisbylla, M.P., Cromartie, T.H., Dagarin, D.P., Howard, S.W., McLean Provan, W., Ellis, M.K., Fraser, T., and Mutter, L.C., *The Discovery and Structural Requirements of Inhibitors of* p-*Hydroxyphenylpyruvate Dioxygenase.* Weed Science, 1997. **45**(5): p. 601-609.

121. Mitchell, G., Bartlett, D.W., Fraser, T.E.M., Hawkes, T.R., Holt, D.C., Townson, J.K., and Wichert, R.A., *Mesotrione: A New Selective Herbicide for Use in Maize.* Pest Management Science, 2001. **57**(2): p. 120-128.

122. Dayan, F.E., Howell, J.L., Marais, J.P., Ferreira, D., and Koivunen, M., *Manuka Oil, a Natural Herbicide with Preemergence Activity.* Weed Science, 2011. **59**(4): p. 464-469.

123. Duke, S.O., Dayan, F.E., Romagni, J.G., and Rimando, A.M., *Natural Products as Sources of Herbicides: Current Status and Future Trends.* Weed Research, 2000. **40**(1): p. 99-111.

124. Romagni, J.G., Allen, S.N., and Dayan, F.E., *Allelopathic Effects of Volatile Cineoles on Two Weedy Plant Species.* Journal of Chemical Ecology, 2000. **26**(1): p. 303-313.

125. Jones, R.G., *Cinmethylin - a New Herbicide Developed for Use in Rice*, in *Pest Management in Rice*, B.T. Grayson, M.B. Green, and L.G. Copping, Editors. 1990, Elsevier Applied Science: London - New York. p. 349-357.

126. Grossmann, K., Hutzler, J., Tresch, S., Christiansen, N., Looser, R., and Ehrhardt, T., *On the Mode of Action of the Herbicides Cinmethylin and 5-Benzyloxymethyl-1, 2-Isoxazolines: Putative Inhibitors of Plant Tyrosine Aminotransferase.* Pest Management Science, 2012. **68**(3): p. 482-492.

127. *Pesticide Information Profile: Endothall.* EXTOXNET: The Extension Toxicology Network, 1995.

128. Matsuzawa, M., Graziano, M.J., and Casida, J.E., *Endothall and Cantharidin Analogs: Relation of Structure to Herbicidal Activity and Mammalian Toxicity.* Journal of Agricultural and Food Chemistry, 1987. **35**(5): p. 823-829.

129. Tresch, S., Schmotz, J., and Grossmann, K., *Probing Mode of Action in Plant Cell Cycle by the Herbicide Endothall, a Protein Phosphatase Inhibitor.* Pesticide Biochemistry and Physiology, 2011. **99**(1): p. 86-95.

130. Bogran, C.E., Ludwig, S., and Metz, B., *Using Oils as Pesticides.* Texas A&M Agrilife Extension, (E-419 11/06): p. 1-5.

131. Palla, O., *Tecnobiol: A New Formulation Based on Fatty Acids Coming from Vegetal Oils Effective against Grapevine Downy Mildew.* ATTI Giornate Fitopatologiche, 2006. **2**: p. 17-20.

132. Isman, M.B., *Plant Essential Oils for Pest and Disease Management.* Crop Protection, 2000. **19**(8): p. 603-608.

133. Owen, W.J., Yao, C., Myung, K., Kemmitt, G., Leader, A., Meyer, K.G., Bowling, A.J., Slanec, T., and Kramer, V.J., *Biological Characterization of Fenpicoxamid, a New Fungicide with Utility in Cereals and other Crops.* Pest Management Science, 2017. **73**(10): p. 2005-2016.

134. Nakajima, M., Itoi, K., Takamatsu, Y., Sato, S., Furukawa, Y., Furuya, K., Honma, T., Kadotani, J., Kozasa, M., and Haneishi, T., *Cornexistin: A New Fungal Metabolite with Herbicidal Activity.* The Journal of Antibiotics, 1991. **44**(10): p. 1065-1072.

135. Dayan, F.E., Owens, D.K., Watson, S.B., Asolkar, R.N., and Boddy, L.G., *Sarmentine, a Natural Herbicide from* Piper *Species with Multiple Herbicide Mechanisms of Action.* Frontiers in Plant Science, 2015. **6**(222): p. 1-11.

136. Isman, M.B. and Seffrin, R., *Chapter 2: Natural Insecticides from the Annonaceae: A Unique Example for Developing Biopesticides*, in *Advances in Plant Biopesticides*, D. Singh, Editor. 2014, Springer India: New Delhi. p. 21-33.

137. Dabbert, S., Häring, A.M., and Zanoli, R., *Organic Farming: Policies and Prospects* 2004, London - New York: Zed Books.

138. *Anuual Report 2007*, 2008, IFOAM - Organics International: Bonn.

139. *Annual Report 2008*, 2009, IFOAM - Organics International: Bonn.

140. Stockdale, E.A., Lampkin, N.H., Hovi, M., Keatinge, R., Lennartsson, E.K.M., Macdonald, D.W., Padel, S., Tattersall, F.H., Wolfe, M.S., and Watson, C.A., *Agronomic and Environmental Implications of Organic Farming Systems*, in *Advances in Agronomy*, M. Alexander, et al., Editors. 2001, Academic Press: Cambridge, USA. p. 261-327.

141. Rigby, D. and Caceres, D., *Organic Farming and the Sustainability of Agricultural Systems.* Agricultural Systems, 2001. **68**(1): p. 21-40.

142. Lotter, D.W., *Organic Agriculture.* Journal of Sustainable Agriculture, 2003. **21**(4): p. 59-128.

143. Nandwani, D. and Nwosisi, S., *Global Trends in Organic Agriculture*, in *Organic Farming for Sustainable Agriculture*, D. Nandwani, Editor. 2016, Springer International Publishing Switzerland: Cham. p. 1-35.

144. Vogt, G., *The Origins of Organic Farming*, in *Organic Farming: An International History*, W. Lockeretz, Editor. 2007, CABI: Wallingford, Oxfordshire. p. 1-8.

145. Lockeretz, W., *What Explains the Rise of Organic Farming?*, in *Organic Farming: An International History*, W. Lockeretz, Editor. 2007, CABI: Wallingford, Oxfordshire. p. 1-8.

146. *Organic Garming 'Scientific Sonsense'.* Washington Post, 28 February 1974.

147. Lockeretz, W., Shearer, G., and Kohl, D.H., *Organic Farming in the Corn Belt.* Science, 1981. **211**(4482): p. 540-547.

148. Willer, H. and Lernoud, J., *The World of Organic Agriculture 2017: Summary*, in *The World of Organic Agriculture. Statistics and Emerging Trends 2017*, H. Willer and J. Lernoud, Editors. 2017, Research Institute of Organic Agriculture (FiBL) and IFOAM - Organics International: Frick - Bonn.

149. Reganold, J.P. and Wachter, J.M., *Organic Agriculture in the Twenty-First Century.* Nature Plants, 2016. **2**(15221): p. 1-8.

150. Goklany, I.M., *The Ins and Outs of Organic Farming.* Science, 2002. **298**(5600): p. 1889-1890.

151. Badgley, C., Moghtader, J., Quintero, E., Zakem, E., Chappell, M.J., Aviles-Vazquez, K., Samulon, A., and Perfecto, I., *Organic Agriculture and the Global Food Supply.* Renewable Agriculture and Food Systems, 2007. **22**(2): p. 86-108.

152. Forster, D., Adamtey, N., Messmer, M.M., Pfiffner, L., Baker, B., Huber, B., and Niggli, U., *Organic Agriculture—Driving Innovations in Crop Research*, in *Agricultural Sustainability: Progress and Prospects in Crop Research*, G.S. Bhullar and N.K. Bhullar, Editors. 2012, Elsevier Science: Amsterdam.

153. Pimentel, D., Harvey, C., Resosudarmo, P., Sinclair, K., Kurz, D., McNair, M., Crist, S., Shpritz, L., Fitton, L., and Saffouri, R., *Environmental and Economic Costs of Soil Erosion and Conservation Benefits.* Science, 1995. **267**(5201): p. 1117-1122.

154. Naylor, R.L., *Energy and Resource Constraints on Intensive Agricultural Production.* Annual Review of Energy and the Environment, 1996. **21**(1): p. 99-123.

155. Hole, D.G., Perkins, A.J., Wilson, J.D., Alexander, I.H., Grice, P.V., and Evans, A.D., *Does Organic Farming Benefit Biodiversity?* Biological Conservation, 2005. **122**(1): p. 113-130.

156. Lairon, D., *Nutritional Quality and Safety of Organic Food. A Review.* Agronomy for sustainable development, 2010. **30**(1): p. 33-41.

157. Connor, D.J., *Organic Agriculture Cannot Feed the World.* Field Crops Research, 2008. **106**(2): p. 187-190.

158. de Ponti, T., Rijk, B., and van Ittersum, M.K., *The Crop Yield Gap Between Organic and Conventional Agriculture.* Agricultural Systems, 2012. **108**: p. 1-9.

159. Leifeld, J., *How Sustainable is Organic Farming?* Agriculture, Ecosystems & Environment, 2012. **150**: p. 121-122.

160. Edwards-Jones, G. and Howells, O., *The Origin and Hazard of Inputs to Crop Protection in Organic Farming Systems: Are they Sustainable?* Agricultural Systems, 2001. **67**(1): p. 31-47.

161. Trewavas, A., *Urban Myths of Organic Farming.* Nature, 2001. **410**(6827): p. 409-410.

162. Niggli, U., Andres, C., Willer, H., and Baker, B.P., *Building a Global Platform for Organic Farming Research, Innovation and Technology Transfer.* Organic Agriculture, 2017. **7**(3): p. 209-224.

163. Borkow, G. and Gabbay, J., *Copper as a Biocidal Tool.* Current Medicinal Chemistry, 2005. **12**(18): p. 2163-2175.

164. Georgopoulos, P.G., Roy, A., Yonone-Lioy, M.J., Opiekun, R.E., and Lioy, P.J., *Environmental Copper: Its Dynamics and Human Exposure Issues.* Journal of Toxicology and Environmental Health, Part B, 2001. **4**(4): p. 341-394.

165. Kiaune, L. and Singhasemanon, N., *Pesticidal Copper (I) Oxide: Environmental Fate and Aquatic Toxicity*, in *Reviews of Environmental Contamination and Toxicology*, D.M. Whitacre, Editor. 2011, Springer: New York. p. 1-26.

166. Kula, H., *Kupfer und Umwelt: Auswirkungen von Kupfer auf Gewässerorganismen*, in *Alternativen zur Anwendung von Kupfer als Pflanzschutzmittel. 7. Fachgespräch am*

6. Juni 2002 in Berlin-Dahlem. Berichte aus der biologischen Bundesanstalt für Land- und Forstwirtschaft 2003. p. 6-10.

167. Paoletti, M.G., Sommaggio, D., Favretto, M.R., Petruzzelli, G., Pezzarossa, B., and Barbafieri, M., *Earthworms as Useful Bioindicators of Agroecosystem Sustainability in Orchards and Vineyards with Different Inputs.* Applied Soil Ecology, 1998. **10**(1–2): p. 137-150.

168. Rusjan, D., Strlic, M., Pucko, D., and Korosec-Koruza, Z., *Copper Accumulation Regarding the Soil Characteristics in Sub-Mediterranean Vineyards of Slovenia.* Geoderma, 2007. **141**(1-2): p. 111-118.

169. Eijsackers, H., Beneke, P., Maboeta, M., Louw, J.P.E., and Reinecke, A.J., *The Implications of Copper Fungicide Usage in Vineyards for Earthworm Activity and Resulting Sustainable Soil Quality.* Ecotoxicology and Environmental Safety, 2005. **62**(1): p. 99-111.

170. Thrupp, L.A., *Long-term Losses from Accumulation of Pesticide Residues: A Case of Persistent Copper Toxicity in Soils of Costa Rica.* Geoforum, 1991. **22**(1): p. 1-15.

171. Kula, C. and Guske, S., *Auswirkungen von Kupfer auf Bodenorganismen bei langjähriger Anwendung*, in *Alternativen zur Anwendung von Kupfer als Pflanzschutzmittel. 7. Fachgespräch am 6. Juni 2002 in Berlin-Dahlem. Berichte aus der biologischen Bundesanstalt für Land- und Forstwirtschaft*2003. p. 11-16.

172. Speiser, B., Mieves, E., and Tamm, L., *Kupfereinsatz von Schweizer Biobauern in verschiedenen Kulturen.* Agrarforschung Schweiz, 2015. **6**(4): p. 160-165.

173. Kienzle, J., *Einsatz von Kupfer im ökologischen Obstbau*, in *Alternativen zur Anwendung von Kupfer als Pflanzschutzmittel. 7. Fachgespräch am 6. Juni 2002 in Berlin-Dahlem. Berichte aus der biologischen Bundesanstalt für Land- und Forstwirtschaft* 2003. p. 19-20.

174. Gitzel, J. and Kühne, S., *Status Quo der Forschung zu Minimierung und Ersatz kupferhaltiger Pflanzeschutzmittel in Deutschland.* Journal für Kultupflanzen, 2016. **68**(5): p. 125-129.

175. Bucar, F., Wube, A., and Schmid, M., *Natural Product Isolation – How to Get from Biological Material to Pure Compounds.* Natural Product Reports, 2013. **30**(4): p. 525-545.

176. Sticher, O., *Natural Product Isolation.* Natural Product Reports, 2008. **25**(3): p. 517-554.

177. Wolfender, J.-L., *HPLC in Natural Product Analysis: The Detection Issue.* Planta Medica, 2009. **75**(7): p. 719-734.

178. Kwan, E.E. and Huang, S.G., *Structural Elucidation with NMR Spectroscopy: Practical Strategies for Organic Chemists.* European Journal of Organic Chemistry, 2008. **2008**(16): p. 2671-2688.

179. Di Bari, L. and Pescitelli, G., *Chapter 9: Electronic Circular Dichroism*, in *Computational Spectroscopy*, J. Grunenberg, Editor. 2010, Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim. p. 241-277.

180. Berova, N., Di Bari, L., and Pescitelli, G., *Application of Electronic Circular Dichroism in Configurational and Conformational Analysis of Organic Compounds.* Chemical Society Reviews, 2007. **36**(6): p. 914-931.

181. Ladd, M. and Palmer, R., *Structure Determination by X-ray Crystallography*. Fifth ed 2013, New York: Springer.

182. Ash, G., *Downy Mildew of Grape.* The Plant Health Instructor, 2000: p. DOI: 10.1094/PHI-I-2000-1112-01 Updated 2017.

183. Holb, I., Heijne, B., and Tamm, L., *Chapter 5.6: Organic Apple Disease Management*, in *Plant Diseases and Their Management in Organic Agriculture*, M.R. Finckh, A.H.C. van Bruggen, and L. Tamm, Editors. 2015, The American Phytopathological Society: St. Paul. p. 319-334.

184. Vaillancourt, L.J. and Hartman, J.R., *Apple Scab.* The Plant Health Instructor, 2000: p. DOI: 10.1094/PHI-I-2000-1005-01 Updated 2005.

185. Finckh, M.R., Tamm, L., and Bruns, C., *Chapter 5.1: Organic Potato Disease Management*, in *Plant Diseases and Their Management in Organic Agriculture*, M.R. Finckh, A.H.C. Van Bruggen, and L. Tamm, Editors. 2015, The American Phytopathological Society: St. Paul. p. 239-257.

186. Schumann, G.L. and D'Arcy, C.J., *Late Blight of Potato and Tomato.* The Plant Health Instructor, 2000: p. DOI: 10.1094/PHI-I-2000-0724-01 Updated 2005.

187. Potterat, O. and Hamburger, M., *Concepts and Technologies for Tracking Bioactive Compounds in Natural Product Extracts: Generation of Libraries, and Hyphenation of Analytical Processes with Bioassays.* Natural Product Reports, 2013. **30**(4): p. 546-564.

188. Potterat, O., *Targeted Approaches in Natural Product Lead Discovery.* CHIMIA International Journal for Chemistry, 2006. **60**(1-2): p. 19-22.

189. Hamburger, M., *New Approaches in Analyzing the Pharmacological Properties of Herbal Extracts.* Proceedings of the Western Pharmacology Society, 2007. **50**: p. 156-161.

190. *Efficacy Evaluation of Plant Protection Products: Phytotoxicity Assessment.* EPPO Bulletin, 2007. **37**(1): p. 4-10.

191. *Efficacy Evaluation of Fungicides: Plasmopara viticola.* EPPO Bulletin, 2001. **31**(2): p. 313-317.

192. Tehon, L.R. and Stout, G.L., *Epidemic Diseases of Fruit Trees in Illinois 1922-1928.* Illinois Natural History Survey Bulletin, 1930. **18**(3): p. 415-509.

193. *Efficacy Evaluation of Fungicides: Venturia inaequalis and* V. pyrina. EPPO Bulletin, 1990. **20**(3): p. 425-433, Updated in 1996.

194. *Efficacy Evaluation of Plant Protection Products: Design and Analysis of Efficacy Evaluation Trials.* EPPO Bulletin, 2012. **42**(3): p. 367-381.

195. Mabberley, D.J., *Mabberley's Plant-book: A Portable Dictionary of Plants, Their Classification and Uses*. Third ed 2008, Cambridge: Cambridge University Press.

196. Gurib-Fakim, A. and Brendler, T., *Medicinal and Aromatic Plants of Indian Ocean Islands: Madagascar, Comoros, Seychelles and Mascarenes* 2004, Stuttgart: Medpharm.

197. Genaust, H., *Etymologisches Wörterbuch der botanischen Pflanzennamen*. Sixth ed 2017, Hamburg: Nikol Verlag.

198. Xu, Z. and Chang, L., *Juncaceae*, in *Identification and Control of Common Weeds*. 2017, Springer: Singapore. p. 821-834.

199. Guofang, W. and Clemants, S.E., *Juncaceae.* Flora of China, 2000. **24**: p. 44-69.

200. Richards, P.W. and Clapham, A.R., Juncus Effusus *L. (*Juncus Communis β effusus *E. Mey).* Journal of Ecology, 1941. **29**(2): p. 375-380.

201. Moerman, D.E., *Native American Medicinal Plants: An Ethnobotanical Dictionary* 2009, Portland - London: Timber Press.

202. Lavergne, R., *Le Grand Livre des Tisaneurs et Plantes Médicinales Indigènes: L'Ile de la Réunion* 1990, Saint-Denis: Orphie.

203. Tang, W. and Eisenbrand, G., *Handbook of Chinese Medicinal Plants: Chemistry, Pharmacology, Toxicology*. Vol. 1. 2011, Weinheim: Wiley-VCH.

204. Su, X.-H., Yuan, Z.-P., Li, C.-Y., Zhong, Y.-J., Du, H.-J., Wen, Y.-Y., Li, Y.-F., and Liang, B., *Phenanthrenes from* Juncus effusus. Planta Medica, 2013. **79**(15): p. 1447-1452.

205. Kovacs, A., Vasas, A., and Hohmann, J., *Natural Phenanthrenes and their Biological Activity.* Phytochemistry, 2008. **69**(5): p. 1084-1110.

206. Della Greca, M., Fiorentino, A., Monaco, P., Pinto, G., Pollio, A., and Previtera, L., *Action of Antialgal Compounds from* Juncus effusus *L. on* Selenastrum capricornutum. Journal of Chemical Ecology, 1996. **22**(3): p. 587-603.

207. Li, H.-X., Deng, T.-Z., Chen, Y., Feng, H.-J., and Yang, G.-Z., *Isolation and Identification of Phenolic Constituents from* Juncus effusus. Yao xue xue bao = Acta pharmaceutica Sinica, 2007. **42**(2): p. 174-178.

208. Dong-Zhe, J., Zhi-Da, M., Chiou, G.C.Y., Iinuma, M., and Tanaka, T., *Two p-Coumaroyl Glycerides from* Juncus effusus. Phytochemistry, 1996. **41**(2): p. 545-547.

209. Shan, C.-Y., Ye, Y.-H., Jiang, H.-F., and Zhang, J., *Study on Chemical Constituents Isolated from* Juncus effusus. Zhongyaocai = Journal of Chinese Medicinal Materials, 2008. **31**: p. 374-6.

210. Tian, X.-J., Li, H.-X., Chen, Y., Wang, X.-Q., and Yang, G.-Z., *Studies on the Chemical Constituents of* Juncus effusus *L.* Lishizhen Medicine and Materia Medica Research, 2007. **18**: p. 2121-2122.

211. Corsaro, M.M., Della Greca, M., Fiorentino, A., Monaco, P., and Previtera, L., *Cycloartane Glucosides from* Juncus effusus. Phytochemistry, 1994. **37**(2): p. 515-519.

212. Della Greca, M., Fiorention, A., Monaco, P., and Previtera, L., *Cycloartane Triterpenes from* Juncus effusus. Phytochemistry, 1994. **35**(4): p. 1017-1022.

213. Della Greca, M., Fiorentino, A., Monaco, P., Previtera, L., and Sorrentino, M., *Antialgal Phenylpropane Glycerides from* Juncus effusus. Natural Product Letters, 1998. **12**(4): p. 263-270.

214. Mody, N.V., Mahmoud, I.I., Finer-Moore, J., and Pelletier, S.W., *Constituents of* Juncus effusus*: The X-ray Analysis of Effusol Diacetate.* Journal of Natural Products, 1982. **45**(6): p. 733-737.

215. Wang, Y.-G., Wang, Y.-L., Zhai, H.-F., Liao, Y.-J., Zhang, B., and Huang, J.-M., *Phenanthrenes from* Juncus effusus *with Anxiolytic and Sedative Activities.* Natural Product Research, 2012. **26**(13): p. 1234-1239.

216. Hanawa, F., Okamoto, M., and Towers, G.H.N., *Antimicrobial DNA-binding Photosensitizers from the Common Rush,* Juncus effusus. Photochemistry and Photobiology, 2002. **76**(1): p. 51-56.

217. Della Greca, M., Fiorentino, A., Mangoni, L., Molinaro, A., Monaco, P., and Previtera, L., *Cytotoxic 9,10-Dihydrophenanthrenes from* Juncus effusus *L.* Tetrahedron, 1993. **49**(16): p. 3425-3432.

218. Della Greca, M., Fiorentino, A., Mangoni, L., Molinaro, A., Monaco, P., and Previtera, L., *9,10-Dihydrophenanthrene Metabolites from* Juncus effusus *L.* Tetrahedron Letters, 1992. **33**(36): p. 5257-5260.

219. Evans, W.C. and Evans, D., *Trease and Evans Pharmacognosy*. Fifteenth ed 2002, Philadelphia: W.B. Saunders.

220. Fernandez, C.G., *Chapter 10: Benzoin, a Resin Produced by Styrax trees in North Sumatra Province, Indonesia*, in *Forest Products, Livelihoods and Conservation*, K. Kuster and B. Belcher, Editors. 2004, Center for International Forestry Research: Jakarta.

221. Hänsel, R., Keller, K., Rimpler, H., and Schneider, G., *Hagers Handbuch der Pharmazeutischen Praxis: Band 6: Drogen P-Z* 1994, Berlin - Heidelberg: Springer-Verlag.

222. Katz, E., Garcia, C., and Goloubinoff, M., *Chapter 23: Sumatra benzoin (*Styrax *spp.)*, in *Tapping the Green Market: Certification and Management of Non-timber Forest Products*, P. Shanley, et al., Editors. 2002, Earthscan Publications: London.

223. Pinyopusarerk, K., Styrax tonkinensis*: Taxonomy, Ecology, Silviculture and Uses*. Technical Reports Series. Vol. 31. 1994, Canberra: Australian Centre for International Agricultural Research (ACIAR).

224. Langenheim, J.H., *Plant Resins: Chemistry, Evolution, Ecology, and Ethnobotany* 2003, Portland - Cambridge: Timber Press.

225. Zhao, Z., *An Illustrated Chinese Materia Medica in Hong Kong* 2004, Hong Kong: School of Chinese Medicine, Hong Kong Baptist University.

226. Khan, I.A. and Abourashed, E.A., *Leung's Encyclopedia of Common Natural Ingredients Used in Food, Drugs, and Cosmetics*. Third ed 2010, Hoboken: John Wiley & Sons.

227. Robbers, J.E., Speedie, M.K., and Tyler, V.E., *Pharmacognosy and Pharmacobiotechnology* 1996, Baltimore: Williams & Wilkins.

228. Hovaneissian, M., Archier, P., Mathe, C., and Vieillescazes, C., *Contribution de la Chimie Analytique à l'Etude des Exsudats Végétaux Styrax, Storax et Benjoin.* Comptes Rendus Chimie, 2006. **9**(9): p. 1192-1202.

229. Coppen, J.J.W., *Gums, Resins and Latexes of Plant Origin*. Non-wood forest products. Vol. 6. 1995, Rome: Food and Agriculture Organization of the United Nations (FAO).

230. Burger, P., Casale, A., Kerdudo, A., Michel, T., Laville, R., Chagnaud, F., and Fernandez, X., *New Insights in the Chemical Composition of Benzoin Balsams.* Food Chemistry, 2016. **210**: p. 613-622.

231. Hartwich, C., *Ueber die Siam Benzoe.* Apotheker-Zeitung, 1913. **28**: p. 685-687 and 698-699.

232. Dryander, J., *Botanical Description of the Benjamin Tree of Sumatra.* Philosophical transactions of the Royal Society of London, 1787. **77**: p. 307-309.

233. Perkins, J.R., *Beiträge zur Kenntis der Styracaceae.* Botanische Jahrbücher fur Systematik, Pflanzengeschichte und Pflanzengeographie, 1902. **31**: p. 484-488.

234. *Pharmacopoea Helvetica 9* 2003, Bern: Swissmedic.

235. Stahl, E. and Schild, W., *Drogenanalyse II: Inhaltsstoffe und Isolierung*. Pharmazeutische Biologie. Vol. 4. 1981, Stuttgart - New York: Gustav Fischer Verlag.

236. *European Pharmacopoeia 7.0*. Vol. 1. 2011, Strasbourg: European Directorate for the Quality of Medicines & HealthCare of the Council of Europe (EDQM).

237. *British pharmacopoeia 2016*. Vol. 4. 2015, London: The Stationary Office.

238. Rätsch, C., *Räucherstoffe, der Atem des Drachen*. Fourth ed 2006, Aarau: AT-Verlag.

239. *Naturals Marketing Sheets: Benzoin*, 2016, Firmenich: Perf V5.

240. Danam, R., DiNovi, M., and Schlatter, J., Benzoe tonkinensis, in *Safety Evaluation of Certain Food Additives and Contaminants / WHO Food Additives Series: 65*. 2012, Seventy-Fourth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA): Geneva. p. 87-100.

241. Rath, S. and Wallin, H., Benzoe tonkinensis, in *Chemical and Technical Assessment (CTA)* 2014, Seventy-ninth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA): Geneva. p. 1-8.

242. Wang, F., Hua, H.-M., Bian, X., Pei, Y.-H., and Jing, Y.-K., *Two New Aromatic Compounds from the Resin of* Styrax tonkinensis *(Pier.) Craib.* Journal of Asian Natural Products Research, 2006. **8**(1-2): p. 137-141.

243. van Wyk, B.-E., Wink, C., and Wink, M., *Handbuch der Arzneipflanzen: Ein illustrierter Leitfaden* 2004, Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH.

244. Pauletti, P.M., Teles, H.L., Silva, D.H.S., Araujo, A.R., and Bolzani, V.S., *The Styracaceae.* Brasilian Journal of Pharmacognosy, 2006. **16**(4): p. 576-590.

245. Nitta, A., Tani, S., Sakamaki, E., and Saito, Y., *On the Source and Evaluation of Benzoin.* Yakugaku Zasshi, 1984. **104**(6): p. 592-600.

246. *Compendium of Food Additive Specifications*. FAO JECFA Monographs Vol. 16. 2014, Rome: Seventy-ninth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

247. Wang, F., Hua, H., Pei, Y., Chen, D., and Jing, Y., *Triterpenoids from the Resin of* Styrax tonkinensis *and Their Antiproliferative and Differentiation Effects in Human Leukemia HL-60 Cells.* Journal of Natural Products, 2006. **69**(5): p. 807-810.

248. Hovaneissian, M., Archier, P., Mathe, C., Culioli, G., and Vieillescazes, C., *Analytical Investigation of Styrax and Benzoin Balsams by HPLC-PAD-fluorimetry and GC-MS.* Phytochemical Analysis, 2008. **19**(4): p. 301-310.

249. Pastorova, I., de Koster, C.G., and Boon, J.J., *Analytical Study of Free and Ester Bound Benzoic and Cinnamic Acids of Gum Benzoin Resins by GC–MS and HPLC–frit FAB–MS.* Phytochemical Analysis, 1997. **8**(2): p. 63-73.

250. Tuntipaleepun, M., Chakthong, S., Ponglimanont, C., Plodpai, P., and Voravuthikunchai, S.P., *Antifungal and Cytotoxic Substances from the Stem Barks of* Desmos chinensis. Chinese Chemical Letters, 2012. **23**(5): p. 587-590.

251. Blaschek, W., Hänsel, R., Keller, K., Reichling, J., Rimpler, H., and Schneider, G., *Hagers Handbuch der Pharmazeutischen Praxis: Folgeband 3: Drogen L-Z* 1998, Berlin- Heidelberg: Springer-Verlag.

252. Patocka, J., Jakl, J., and Strunecká, A., *Expectations of Biologically Active Compounds of the Genus* Magnolia *in Biomedicine.* Journal of Applied Biomedicine, 2006. **4**: p. 171-178.

253. Sargent, C.S. and Wilson, E.H., *Plantae Wilsonianae: An Enumeration of the Woody Plants Collected in Western China for the Arnold Arboretum of Harvard University during the years 1907, 1908, and 1910*. Vol. 1. 1913, Cambridge: University Press.

254. Shu, X., Yang, X., and Yang, Z., *Variation in Seed and Seedling Traits among Fifteen Chinese Provenances of* Magnolia officinalis. Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 2012. **40**(2): p. 274-283.

255. Chang, H.-M. and But, P.P.-H., *Pharmacology and Applications of Chinese Materia Medica*. Vol. 2. 1996, Singapore: World Scientific.

256. Tang, W. and Eisenbrand, G., *Handbook of Chinese Medicinal Plants: Chemistry, Pharmacology, Toxicology*. Vol. 2. 2011, Weinheim: Wiley-VCH.

257. Russo, E.B. and Hou, J., *The Healing Power of Chinese Herbs and Medicinal Recipes* 2012, Abingdon-on-Thames: Taylor & Francis.

258. Yan, R., Wang, W., Guo, J., Liu, H., Zhang, J., and Yang, B., *Studies on the Alkaloids of the Bark of* Magnolia officinalis*: Isolation and On-line Analysis by HPLC-ESI-MSn.* Molecules, 2013. **18**(7): p. 7739-7750.

259. Shen, C.-C., Ni, C.-L., Shen, Y.-C., Huang, Y.-L., Kuo, C.-H., Wu, T.-S., and Chen, C.-C., *Phenolic Constituents from the Stem Bark of* Magnolia officinalis. Journal of Natural Products, 2009. **72**(1): p. 168-171.

260. Yahara, S., Nishiyori, T., Kohda, A., Nohara, T., and Nishioka, I., *Isolation and Characterization of Phenolic Compounds from* Magnoliae Cortex *Produced in China.* Chemical & Pharmaceutical Bulletin, 1991. **39**(8): p. 2024-2036.

261. Rios, J.L., Giner, R.M., and Prieto, J.M., *New Findings on the Bioactivity of Lignans*, in *Studies in Natural Products Chemistry, Vol. 26*, A. ur Rahman, Editor. 2002, Elsevier: Amsterdam. p. 183-292.

262. Bang, K.H., Kim, Y.K., Min, B.S., Na, M.K., Rhee, Y.H., Lee, J.P., and Bae, K.H., *Antifungal Activity of Magnolol and Honokiol.* Archives of Pharmacal Research, 2000. **23**(1): p. 46-49.

263. Bai, X., Cerimele, F., Ushio-Fukai, M., Waqas, M., Campbell, P.M., Govindarajan, B., Der, C.J., Battle, T., Frank, D.A., Ye, K., Murad, E., Dubiel, W., Soff, G., and Arbiser, J.L., *Honokiol, a Small Molecular Weight Natural Product, Inhibits Angiogenesis* in Vitro *and Tumor Growth* in Vivo. The Journal of Biological Chemistry, 2003. **278**(37): p. 35501-35507.

264. Chang, B.S., Lee, Y.M., Ku, Y., Bae, K.H., and Chung, C.P., *Antimicrobial Activity of Magnolol and Honokiol against Periodontopathic Microorganisms.* Planta Medica, 1998. **64**(4): p. 367-369.

265. Ho, J.H.-C. and Hong, C.-Y., *Cardiovascular Protection of Magnolol: Cell-Type Specificity and Dose-Related Effects.* Journal of Biomedical Science, 2012. **19**(70): p. 1-9.

266. Ho, K.-Y., Tsai, C.-C., Chen, C.-P., Huang, J.-S., and Lin, C.-C., *Antimicrobial Activity of Honokiol and Magnolol Isolated from* Magnolia officinalis. Phytotherapy Research, 2001. **15**(2): p. 139-141.

267. Ikeda, K. and Nagase, H., *Magnolol has the Ability to Induce Apoptosis in Tumor Cells.* Biological & Pharmaceutical Bulletin, 2002. **25**(12): p. 1546-1549.

268. Lan, K.-H., Wang, Y.-W., Lee, W.-P., Lan, K.-L., Tseng, S.-H., Hung, L.-R., Yen, S.-H., Lin, H.-C., and Lee, S.-D., *Multiple Effects of Honokiol on the Life Cycle of Hepatitis C Virus.* Liver International, 2012. **32**(6): p. 989-997.

269. Li, X., Fang, Q., Lin, J., Yuan, Z., Han, L., and Gao, Y., *Chemistry Study on Protective Effect against·OH-Induced DNA Damage and Antioxidant Mechanism of* Cortex Magnoliae officinalis. Bulletin of Korean Chemical Society, 2014. **35**(1): p. 117-122.

270. Lin, Y.-R., Chen, H.-H., Ko, C.-H., and Chan, M.-H., *Neuroprotective Activity of Honokiol and Magnolol in Cerebellar Granule Cell Damage.* European Journal of Pharmacology, 2006. **537**(1–3): p. 64-69.

271. Park, J., Lee, J., Jung, E., Park, Y., Kim, K., Park, B., Jung, K., Park, E., Kim, J., and Park, D., In vitro *Antibacterial and Anti-Inflammatory Effects of Honokiol and Magnolol against* Propionibacterium *sp.* European Journal of Pharmacology, 2004. **496**(1–3): p. 189-195.

272. Saito, J., Sakai, Y., and Nagase, H., In Vitro *Anti-Mutagenic Effect of Magnolol against Direct and Indirect Mutagens.* Mutation Research, 2006. **609**(1): p. 68-73.

273. Woodbury, A., Yu, S.P., Wei, L., and Garcia, P., *Neuro-Modulating Effects of Honokiol: A Review.* Frontiers in Neurology, 2013. **4**(130): p. 1-6.

274. Clark, A.M., El-Feraly, A.S., and Li, W.-S., *Antimicrobial Activity of Phenolic Constituents of* Magnolia grandiflora *L.* Journal of Pharmaceutical Sciences, 1981. **70**(8): p. 951-952.

275. Lee, Y.-J., Lee, Y.M., Lee, C.-K., Jung, J.K., Han, S.B., and Hong, J.T., *Therapeutic Applications of Compounds in the* Magnolia *Family.* Pharmacology & Therapeutics, 2011. **130**(2): p. 157-176.

276. Poivre, M. and Duez, P., *Biological Activity and Toxicity of the Chinese Herb* Magnolia officinalis *Rehder & E. Wilson (Houpo) and its Constituents.* Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology), 2017. **18**(3): p. 194-214.

277. Mori, M., Aoyama, M., and Doi, S., *Antifungal Constituents in the Bark of* Magnolia obovata *Thunb.* Holz als Roh- und Werkstoff, 1997. **55**(2-4): p. 275-278.

278. Choi, N.H., Choi, G.J., Min, B.S., Jang, K.S., Choi, Y.H., Kang, M.S., Park, M.S., Choi, J.E., Bae, B.K., and Kim, J.C., *Effects of Neolignans from the Stem Bark of* Magnolia obovata *on Plant Pathogenic Fungi.* Journal of Applied Microbiology, 2009. **106**(6): p. 2057-2063.

279. Yoon, M.-Y., Choi, Y.H., Kang, M.S., Lee, J.H., Han, S.S., Myoung, I.S., Han, B.S., Choi, G.J., Jang, K.S., and Kim, J.-C., *Disease Control Efficacy of the Extract of* Magnolia officinalis *against Perilla and Zoysiagrass Rusts.* Research in Plant Disease, 2013. **19**(1): p. 45-48.

280. Kim, J.C., Choi, G.J., Choi, Y.H., Jang, G.S., Min, B.S., Bae, G.H., and Yoon, U.J., *Composition Containing Neolignan Compound or* Magnolia *Extract that Contains the Neolignan Compound and Used for Controlling Plant Diseases, and Method for Controlling Plant Diseases by Using this Composition*, 2009, Korea Research Institute of Chemical Technology: South Korea Patent 2009020870.

281. Grandtner, M.M., *Elsevier's Dictionary of Trees: Volume 1: North America* 2005, Amsterdam: Elsevier Science.

282. Mora, F.D., Alpan, L., McCracken, V.J., and Nieto, M., *Chemical and Biological Aspects of the Genus* Verbesina*.* The Natural Products Journal, 2013. **3**(2): p. 140-150.

283. Robinson, B.L. and Greenman, J.M., *Synopsis of the Genus* Verbesina*, with an Analytical Key to the Species.* Contributions from the Gray Herbarium of Harvard University, 1899. **16**: p. 534-566.

284. *Verbesina lanata*. http://tropicos.org (accessed 13.06.17).

285. Mora, F.D., Alpan, L., de Tommasi, N., McCracken, V.J., and Nieto, M., *New Antibacterial Germacrene from* Verbesina negrensis*.* Planta Medica, 2013. **79**(8): p. 707-710.

286. Giovannini, P. and Heinrich, M., *Xki Yoma' (Our Medicine) and Xki Tienda (Patent Medicine)—Interface between Traditional and Modern Medicine among the Mazatecs of Oaxaca, Mexico.* Journal of Ethnopharmacology, 2009. **121**(3): p. 383-399.

287. Lobitz, G., Heilmann, J., Zschocke, S., Tamayo-Castillo, G., Bauer, R., and Merfort, I., *Bornyl Cinnamate Derivatives with Anti-inflammatory Activity from* Verbesina turbacensis*. Derivados del Cinamato de Bornilo con Actividad Antiinflamatoria de* Verbesina turbacensis. Pharmaceutical and Pharmacological Letters., 1998. **8**(3): p. 115-118.

288. Campbell, T.N., *Medicinal Plants used by Choctaw, Chickasaw, and Creek Indians in the Early Nineteenth Century.* Journal of the Washington Academy of Sciences, 1951. **41**(9): p. 285-290.

289. Bushnell, D.I., *The Choctaw of Bayou Lacomb, St. Tammany Parish, Louisiana*. Vol. 48. 1909: US Government Printing Office.

290. Sturtevant, W., *The Mikasuki Seminole: Medical Beliefs and Practices. Yale University*, 1954, PhD Thesis: 193.

291. Stevenson, M.C., *Ethnobotany of the Zuñi Indians.* Annual Report of the Bureau of American Ethnology, 1908-1909. **30**: p. 31-102.

292. Vestal, P.A., *The Ethnobotany of the Ramah Navaho.* Papers of the Peabody Museum of American Archaeology and Ethnology, 1952. **40**(4): p. 1-94.

293. Elmore, F.H., *Ethnobotany of the Navajo* 1944, Albuquerque: University of New Mexico Press.

294. Whiting, A.F., *Ethnobotany of the Hopi* 1939, Flagstaff: Northern Arizona Society of Science and Art.

295. Wyman, L.C. and Harris, S.K., *The Ethnobotany of the Kayenta Navaho: An Analysis of the John and Louisa Wetherill Ethnobotanical Collection*. University of New Mexico publications in biology. Vol. 5. 1951, Albuquerque: The University of New Mexico Press.

296. Bruno-Colmenárez, J., Amaro-Luis, J.M., Ramírez, I., Delgado-Méndez, P., and Díaz de Delgado, G., *Absolute Configuration of Two Eudesmane Derivatives from* Verbesina turbacensis *H.B.K.* Journal of Chemical Crystallography, 2010. **40**(2): p. 110-115.

297. Wu, Q.-X., Shi, Y.-P., and Jia, Z.-J., *Eudesmane Sesquiterpenoids from the Asteraceae Family.* Natural Products Reports, 2006. **23**(5): p. 699-734.

298. Jiang, Z.D., *Antifungal Agents*, 1999, Millenium Pharmaceuticals, Inc.: U. S. Patent 5,917,084.

299. Jaramillo, T.S., *48. Myristicaceae*. Flora of Ecuador, ed. P. Muriel and H. Balslev. Vol. 72. 2004, Göteborg: Botanical Institute, Göteborg University.

300. *Iryanthera* spp. http://tropicos.org (accessed 06.11.17).

301. Gentry, A.H., *Additional Panamanian Myristicaceae.* Annals of the Missouri Botanical Garden, 1975. **62**(2): p. 474-479.

302. *Iryanthera megistocarpa*. http://tropicos.org (accessed 07.11.17).

303. Gottlieb, O.R., *Chemical Studies on Medicinal Myristicaceae from Amazonia.* Journal of Ethnopharmacology, 1979. **1**(4): p. 309-323.

304. Grandtner, M.M. and Chevrette, J., *Dictionary of Trees, Volume 2: South America: Nomenclature, Taxonomy and Ecology* 2013, Amsterdam: Elsevier Science.

305. Schultes, R.E. and Holmstedt, B., *Comments on Poisonous Plants from Tropical New World. 8. Miscellaneous Plants of South-America.* Lloydia, 1971. **34**: p. 61-78.

306. Lopez, A., Hudson, J.B., and Towers, G.H.N., *Antiviral and Antimicrobial Activities of Colombian Medicinal Plants.* Journal of Ethnopharmacology, 2001. **77**(2): p. 189-196.

307. Diaz D, P.P. and De Diaz, A.M.P., *Diarylpropanes from the Wood of* Iryanthera grandis. Phytochemistry, 1986. **25**(10): p. 2395-2397.

308. Silva, D.H.S., Cavalheiro, A.J., Yoshida, M., and Gottlieb, O.R., *Flavonolignoids from fruits of* Iryanthera grandis. Phytochemistry, 1995. **38**(4): p. 1013-1016.

309. Vieira, P.C., Gottlieb, O.R., and Gottlieb, H.E., *Tocotrienols from* Iryanthera grandis. Phytochemistry, 1983. **22**(10): p. 2281-2286.

310. Braz Filho, R., Da Silva, M.S., and Gottlieb, O.R., *Flavonoids from* Iryanthera laevis. Phytochemistry, 1980. **19**(6): p. 1195-1197.

311. Garzon N., L., Cuca S., L.E., Martinez V., J.C., Yoshida, M., and Gottlieb, O.R., *Flavonolignoid from the Fruit of* Iryanthera laevis. Phytochemistry, 1987. **26**(10): p. 2835-2837.

312. Conserva, L.M., Yoshida, M., Gottlieb, O.R., Martinez V., J.C., and Gottlieb, H.E., *Iryantherins, Lignoflavonoids of Novel Structural Types from the Myristicaceae.* Phytochemistry, 1990. **29**(12): p. 3911-3918.

313. Lopes, N.P., Silva, D.H.S., Kato, M.J., and Yoshida, M., *Butanolides as a Common Feature of* Iryanthera lancifolia *and* Virola surinamensis. Phytochemistry, 1998. **49**(5): p. 1405-1410.

314. Silva, D.H.S., Davino, S.C., Barros, S.B.d.M., and Yoshida, M., *Dihydrochalcones and Flavonolignans from* Iryanthera lancifolia. Journal of Natural Products, 1999. **62**(11): p. 1475-1478.

315. Mesa-Siverio, D., Machin, R.P., Estevez-Braun, A., Ravelo, A.G., and Lock, O., *Structure and Estrogenic Activity of New Lignans from* Iryanthera lancifolia. Bioorganic & Medicinal Chemistry, 2008. **16**(6): p. 3387-3394.

316. Ming, D.S., Lopez, A., Hillhouse, B.J., French, C.J., Hudson, J.B., and Towers, G.H.N., *Bioactive Constituents from* Iryanthera megistophylla. Journal of Natural Products, 2002. **65**(10): p. 1412-1416.

317. Motter Magri, F.M., Kato, M.J., and Yoshida, M., *Butanolides and a Neolignan from the Fruits of* Iryanthera paraensis *Huber.* Phytochemistry, 1996. **43**(3): p. 669-671.

318. Silva, D.H.S., Yoshida, M., and Kato, M.J., *Flavonoids from* Iryanthera sagotiana. Phytochemistry, 1997. **46**(3): p. 579-582.

319. Conserva, L.M., Yoshida, M., and Gottlieb, O.R., *Diarylpropanes from* Iryanthera ulei. Phytochemistry, 1990. **29**(12): p. 3986-3988.

320. Bernal, F.A., Cuca-Suarez, L.E., Yamaguchi, L.F., and Coy-Barrera, E.D., *LC-DAD-UV and LC-ESI-MS-based Analyses, Antioxidant Capacity, and Antimicrobial Activity of a Polar Fraction from* Iryanthera ulei *Leaves.* Records of Natural Products, 2013. **7**(2): p. 152.

321. Vieira, P.C., Yoshida, M., Gottlieb, O.R., Paulino Filho, H.F., Nagem, T.J., and Braz Filho, R., *γ-Lactones from* Iryanthera *species.* Phytochemistry, 1983. **22**(3): p. 711-713.

322. Silva, D.H.S., Pereira, F.C., Zanoni, M.V.B., and Yoshida, M., *Lipophyllic Antioxidants from* Iryanthera juruensis *Fruits.* Phytochemistry, 2001. **57**(3): p. 437-442.

323. Silva, D.H.S., Zhang, Y., Santos, L.A., Bolzani, V.S., and Nair, M.G., *Lipoperoxidation and Cyclooxygenases 1 and 2 Inhibitory Compounds from* Iryanthera juruensis*.* Journal of Agricultural and Food Chemistry, 2007. **55**(7): p. 2569-2574.

324. Morais, S.K.R., Teixeira, A.F., dos S. Torres, Z.E., Nunomura, S.M., Yamashiro-Kanashiro, E.H., Lindoso, J.A.L., and Yoshida, M., *Biological Activities of Lignoids from Amazon Myristicaceae Species:* Virola michelii*,* V. mollissima*,* V. pavonis *and* Iryanthera juruensis*.* Journal of the Brazilian Chemical Society, 2009. **20**(6): p. 1110-1118.

325. Braz Filho, R., Diaz D, P.P., and Gottlieb, O.R., *Tetronic Acid and Diarylpropanes from* Iryanthera elliptica*.* Phytochemistry, 1980. **19**(3): p. 455-459.

326. de Almeida, M.E.L., Braz Filho, R., von Bülow, M.V., Correa, J.J.L., Gottlieb, O.R., Maia, J.G.S., and da Silva, M.S., *Diarylpropanoids from* Iryanthera polyneura*.* Phytochemistry, 1979. **18**(6): p. 1015-1016.

327. de Lima, R.A., Franca, N.C., Diaz Diaz, P., and Gottlieb, O.R., *Diarylpropanes from* Iryanthera coriacea*.* Phytochemistry, 1975. **14**(8): p. 1831-1833.

328. Martinez V., J.C., *Distribution of Flavonoids in the Myristicaceae.* Phytochemistry, 2000. **55**(6): p. 505-511.

329. Bernal, F.A. and Cuca Suarez, L.E., *Chemical Constituents from* Iryanthera ulei *Warb.* Biochemical Systematics and Ecology, 2009. **37**(6): p. 772-775.

330. Villamil Z., E., Cuca S., L.E., and Martinez V., J.C., *Partial Study of the Bark from* Iryanthera laevis*.* Spectroscopy, 1988. **6**(3-4): p. 157-165.

331. Martinez, V., Juan, C., Cuca, S., and Luis, E., *Dihidrochalconas en Corteza de* Iryanthera laevis*.* Revista Colombiana de Química, 1989. **18**(1-2): p. 37-46.

332. Martinez V., J.C. and Cuca S., L.E., *Chemical Studies of Myristicaceae Species of the Colombian Amazon*, in *Chemistry of the Amazon*. 1995, American Chemical Society: Washington DC. p. 116-123.

333. Aponte, J.C., Vaisberg, A.J., Rojas, R., Caviedes, L., Lewis, W.H., Lamas, G., Sarasara, C., Gilman, R.H., and Hammond, G.B., *Isolation of Cytotoxic Metabolites from Targeted Peruvian Amazonian Medicinal Plants.* Journal of Natural Products, 2008. **71**(1): p. 102-105.

334. Somsrisa, J., Meepowpan, P., Krachodnok, S., Thaisuchat, H., Punyanitya, S., Nantasaen, N., and Pompimon, W., *Dihydrochalcones with Antiinflammatory Activity from Leaves and Twigs of* Cyathostemma argenteum*.* Molecules, 2013. **18**(6): p. 6898.

335. Hinrichs-Berger, J. and Brüstle, S., *Die Marssonina-Blattfallkrankheit des Apfels.* Obstbau, 2015. **2**: p. 72-74.

Justine Ramseyer, Pharmacist, PhD

7, Rue Achille-Merguin
CH-2900 Porrentruy
E-mail: justine.ramseyer@gmail.com

Birthdate: 13 March 1988
Nationality: Swiss
Phone: +41 78 611 44 13

PROFILE

Pharmacist specialised in phytochemistry and discovery of new bioactive compounds
Expertise in analytical technologies
Collaborative and communicative team-player, organisation and multi-tasking skills, fast-learner, analytical thinking

PROFESSIONAL EXPERIENCE

10.2013 – 05.2018 **PhD candidate / Teaching Assistant**
Pharmaceutical Biology Division, Pharmaceutical Sciences Department, University of Basel

- Early discovery and development of plant derived products as substitutes for chemical fungicides in agriculture
  Extraction, Chromatography (HPLC-UV-MS-ELSD, CC, TLC, CPC), HPLC-based activity profiling, isolation, quantification, structure elucidation (NMR, HR-MS, ECD)
- Collaboration with the Research Institute for Organic Farming (FiBL)
- Supervision of Master students and foreign visiting researchers
- Teaching assistant in Plant Systematics and Pharmaceutical Biology practical courses for Bachelor students in Pharmaceutical Sciences
- Working trilingually on a daily basis (French, English, German) and in a multicultural environment with team members from four continents
- Achievements: European patent application, publications in international journals, presentations in international conferences

Pharmacist in Public Pharmacies

03.2014 – Present *Milliet Pharmacies, Porrentruy, CH*

03.2013 – 08.2014 *Cattin Pharmacies, Delémont, CH*

05.2012 – 09.2012 *Fortuna Pharmacy, Sinsheim-Hoffenheim, DE*

- Recipient of Leonardo da Vinci Fellowship for Vocational Education

11.2011 – 03.2012 *Milliet Pharmacies, Porrentruy, CH*

EDUCATION

2009 – 2011 **Federal Diploma of Pharmacist and Master Degree in Pharmacy**
School of Pharmacy Geneva-Lausanne, University of Geneva

- 8 months training in a public pharmacy
  Milliet Pharmacies, Porrentruy, CH
- Master thesis
  Pharmacy, University Hospital of Geneva
  Assessment of chemical cross-contamination during the preparation of cytotoxics (capsules and parenteralia)
  Experience in GMPs and aseptic production

2006 – 2009 **Bachelor Degree in Pharmaceutical Sciences**
School of Pharmacy Geneva-Lausanne, University of Geneva

2003 – 2006 **Federal Maturity**
Lycée Cantonal, Porrentruy, CH

- Specific option: Latin
- Supplementary option: Law and Economics

LANGUAGE SKILLS

English: professional working proficiency

German: professional working proficiency

French: native proficiency

TRAININGS

Scientific Writing	MediWrite GmbH	Patenting and Spin-off	Unitectra
Conflict Management	NorthStar Coaching	Project management (16 QHs)	SPOL

AWARDS

Swiss Society of Phytiatry Prize for the best Poster, Fall Meeting	2015
Swiss Society of Phytiatry Travel Grant to attend the 17th International Plant Protection Congress in Berlin	2015
Debiopharm Prize for the best Master exam in Pharmacy	2011

SELECTED PUBLICATIONS

Thuerig B, Ramseyer J, Hamburger M, Oberhänsli T, Potterat O, Schärer H-J, and Tamm L. *Efficacy of a* Juncus effusus *extract on grapevine and apple plants against* Plasmopara viticola *and Venturia* inaequalis*, and identification of the major active constituent.* **Pest Manag. Sci.**, 2016, **72**(9), 1718-1726

Thuerig B, Ramseyer J, Hamburger M, Oberhänsli T, Potterat O, Schärer H-J, and Tamm L. *Antifungal activity of an extract against* Plasmopara viticola*,* Venturia inaequalis*,* Oidium tuckeri*, and* Phytophthora infestans, **European Patent Application**, July 2016

Ramseyer J, Thuerig B, De Mieri M, Schärer H-J, Oberhänsli T, Gupta, MP, Tamm L, Hamburger M, and Potterat O. *Eudesmane sesquiterpenes from* Verbesina lanata *with inhibitory activity against grapevine downy mildew.* **J. Nat. Prod.**, 2017, **80**(12), 3296-3304

Thuerig B, Ramseyer J, Hamburger M, Ludwig M, Oberhänsli T, Potterat O, Schärer H-J, and Tamm L. *Efficacy of a* Magnolia officinalis *bark extract against grapevine downy mildew and apple scab under controlled and field conditions.* **Crop Prot.**, in press

MEMBERSHIPS

Schweizerischer Apothekerverband PharmaSuisse

Centre d'Animation des Pharmaciens de Suisse (CAP)

Society for Medicinal Plant and Natural Product Research (GA)

Swiss Chemical Society (SCS)

Swiss Society of Phytiatry (SSP)

Société Jurassienne d'Emulation Section Scientifique (SJE)

Association Francophone des Etudiants de Bâle (AFEB)

Association des Anciens Etudiants de l'Ecole Romande de Pharmacie (AAEERP)

Fanfares Réunies de Courtemaîche (FRC) and Harmonie Shostakovich (Bassoon player)

LEISURE ACTIVITIES

Music (clarinet and bassoon) and volunteering in the organisation of different social and professional events (such as dinner event for 300 guests, recruiting 60 musicians for extraordinary concerts, etc.)

Skiing, horse riding, hiking

Historical and cultural activities (e. g. three weeks botanical excursion in Baikal region of Russia in summer 2016)